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Human enteric pathogens circulation in the Bogotá region and its impact on the health of vulnerable communities

José Jordan Lozano

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de **Doctor en Salud Pública** de l'*Universidad Nacional de Colombia*

par

José Seir JORDAN LOZANO

(à *Avignon Université* : Ecole doctorale « AGROSCIENCES & SCIENCES » ED 536)

**Transmissions indirectes *via* l'environnement de
pathogènes impliquées dans les gastroentérites
aiguës de l'Homme à/autour de Bogotá (Colombie)**

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Résumé / Abstract / Resumen

Résumé :

Les gastroentérites aiguës affectent chaque année entre un quart et la moitié des personnes dans le Monde. Elles sont causes de morbidité, de mortalité et de coûts de santé importants. Leur transmission directe ou indirecte *via* l'eau, les aliments, l'air ou les surfaces inertes dépend de leur étiologie (virale, bactérienne ou parasitaire) et du contexte local. *Bogotá* et sa région présentent plusieurs spécificités : des eaux usées rejetées en rivière souvent sans ou après seulement un traitement primaire, la mise en décharge des papiers toilettes, couches et protections souillés par les excréments, et une consommation de fruits et légumes faible et limitée à des produits bon marché irrigués par des eaux pouvant être contaminées fécalement. Notre thèse visait à évaluer les flux de certains pathogènes entériques de l'Homme dans l'environnement à proximité de *Bogotá* et à essayer de relier ces flux à la santé de la population.

La thèse a associé trois contributions. Premièrement, une méthode de culture du norovirus humain a été mise au point en utilisant des villosités intestinales isolées de souris comme modèle cellulaire présentant toute la diversité des cellules épithéliales intestinales. Plusieurs concentrations en trypsine ont été testées pour activer les norovirus ; la méthode a été appliquée à des échantillons fécaux et environnementaux. Deuxièmement, les contaminations en *E. coli* et en pathogènes entériques de l'Homme ont été suivies dans des eaux (lixiviat de décharge, eau de ruissellement, rivière, eau d'irrigation, eau potable), des légumes-feuilles mangés crus (blettes) et l'air (au-dessus d'une décharge, en zone rurale, en zone urbaine) dans la région de *Bogotá*. Troisièmement, l'impact des contextes socioéconomiques et des pratiques individuelles (alimentation, hygiène et santé) sur les cas de gastroentérites aiguës a été testé à partir d'enquêtes réalisées dans un district de *Bogotá* et analysées par divers outils (analyse en composante principale, modélisation ...).

Nous avons montré que les villosités intestinales isolées de souris permettent l'infection et la réplication du norovirus humain. Le virus doit être activé avec de la trypsine et a un cycle répliatif moyen de 10 h. Les villosités sont efficaces pour obtenir un matériel biologique abondant et sont idéales pour étudier l'activité biologique du norovirus ou générer des anticorps. Elles ont permis de voir des norovirus non détectés par méthode moléculaire dans certains excréments ou échantillons environnementaux ; les échantillons positifs par méthode

moléculaire ou en immunodot-blot contenaient quasiment tous des norovirus infectieux. Au niveau régional, les rejets d'eaux usées dans les rivières *Bogotá* et *Balsillas* et dans le marais *Tres Esquinas* contaminent le réseau d'irrigation de *La Ramada* au nord-ouest de *Bogotá* en *E. coli* et potentiellement en pathogènes entériques de l'Homme. Les blettes récoltées dans cette zone étaient fortement contaminées, en contraste d'autres zones de culture. Leur contamination évoluait de leur production à leur achat dans les commerces de proximité, les lavages pouvant être contaminants ou décontaminants, les manipulations sur l'étal des marchands étant contaminantes. L'air était souvent contaminé par *E. coli* et par *Shigella* spp., sans pouvoir attribuer à la décharge *Doña Juana* un rôle particulier. La présence de *Shigella* spp. était observée parallèlement dans plus de la moitié des selles des personnes diarrhéiques. Les enquêtes réalisées ont montré que la fréquence annuelle des gastroentérites aiguës diminuait avec l'accroissement de l'âge des personnes ; elle semblait plus faible dans les foyers avec personnes âgées, peut-être en lien avec des pratiques plus strictes en matière d'hygiène, alimentaire notamment. Un modèle de transmission des gastroentérites aiguës distinguant contamination en provenance de l'extérieur des foyers et contaminations entre personnes d'un même foyer n'a pas permis de mettre en évidence de différence nette entre quartiers. Utilisé pour simuler des expériences numériques, il suggère de travailler sur des enquêtes en nombre beaucoup plus élevé.

Mots clés : gastroentérite aiguë, pathogène entérique, virus, bactérie, protozoaire, transmission fécale-orale, transmission indirecte, contamination de l'eau, contamination des légumes-feuilles, contamination de l'air, décharge, maraîchage, irrigation, pratiques alimentaires, pratiques d'hygiène, pratique de santé, conditions socioéconomiques, suivis expérimentaux, modélisation, culture du norovirus humain

Abstract:

Acute gastroenteritis affect between a quarter and a half of people in the World each year. They are responsible for significant morbidity, mortality and healthcare costs. Their direct or indirect transmissions *via* water, food, air or inert surfaces depend on their aetiology (viral, bacterial or parasitic) and the local context. *Bogotá* and its region have several specificities: wastewater are often discharged into rivers without or after primary treatment only, the deposit in landfill of toilet papers and diapers soiled by excrement, and the low consumption of fruits and vegetables largely restricted to a handful of relatively cheap products that may be irrigated by surface freshwaters heavily contaminated with faeces. Our PhD aimed to assess the fluxes of some human enteric pathogens in the region of *Bogotá* and to try to relate these fluxes to the population health.

The PhD combined three contributions. First, a method for culturing the human norovirus has been developed using isolated mouse intestinal villi as a cell model exhibiting the full diversity of intestinal epithelial cells. Several concentrations of trypsin were tested to activate noroviruses; the method was applied to faecal and environmental samples. Second, contamination with *E. coli* and some human enteric pathogens was monitored in water (landfill leachate, runoff water, river, irrigation water, drinking water), leafy vegetables eaten raw (chards) and air (above a landfill, in rural areas, in urban areas) in the *Bogotá* region. Third, the impact of socioeconomic contexts and individual practices (food, hygiene and health) on cases of acute gastroenteritis was assessed from surveys carried out in one district of *Bogotá* and analysed by various tools (principal component analysis, modelling ...).

We have shown that mouse isolated intestinal villi allow the infection and replication of human norovirus. The virus has to be activated with trypsin and has an average replicative cycle of 10 h. Villi are efficient in obtaining abundant biological material and are ideal for studying the biological activity of norovirus or for generating antibodies. They made it possible to see infectious noroviruses not detected by molecular method in several faeces and environmental samples; almost all samples positive by molecular method or immunodot-blot contain infectious noroviruses. At the regional level, the discharges of wastewater in the *Bogotá* and *Balsillas* rivers and in *Tres Esquinas* march contaminate the irrigation network of *La Ramada* area in the northwest of *Bogotá* with *E. coli* and potentially human enteric pathogens. Chards harvested in this area were heavily contaminated, in contrast to other growing areas. Their contamination evolved from their production to their purchase in nearby

stores, washings increasing or decreasing their contamination, and handling on the merchant's stalls increasing contamination. The air was often contaminated with *E. coli* and *Shigella* spp.; it was not possible to detect a particular contribution of the *Doña Juana* landfill in pathogen aerosolization. The presence of *Shigella* spp. was observed in parallel in more than half of the stools of people with diarrhoea. Surveys have shown that the annual frequency of acute gastroenteritis decreases with increasing age; it seemed less common in households with elderly people, possibly due to stricter food hygiene practices. A transmission model of acute gastroenteritis distinguishing contamination from outside the households and contaminations between people in the same households did not show significant differences between neighbourhoods. Used to simulate numerical experiments, it suggests working on much higher numbers of surveys.

Key words: acute gastroenteritis, enteric pathogens, virus, bacteria, protozoa, faecal-oral transmission, indirect transmission, water contamination, leafy vegetable contamination, air contamination, landfill, market gardening, irrigation, food practices, hygiene practices, health practices, socioeconomic conditions, experimental monitoring, modelling, culture of human norovirus.

Resumen:

La gastroenteritis aguda afecta entre una cuarta parte y la mitad de las personas en el mundo cada año. Son responsables de importantes costos de morbilidad, mortalidad y asistencia sanitaria. Sus transmisiones directas o indirectas a través del agua, alimentos, aire o superficies inertes dependen de su etiología (viral, bacteriana o parasitaria) y del contexto local. Bogotá y su región aledaña tienen varias especificidades: las aguas residuales a menudo se vierten a los ríos sin o solo después de un tratamiento primario, el depósito de papel higiénico y pañales sucios con excrementos son dispuestos generalmente en un relleno sanitario, y el bajo consumo de frutas y verduras restringido en gran medida a un puñado de productos relativamente baratos pueden ser irrigados por aguas dulces superficiales muy contaminadas con excrementos. Nuestra tesis doctoral tuvo como objetivo evaluar los flujos de algunos patógenos entéricos humanos en la región de Bogotá y tratar de relacionar estos flujos con la salud de la población.

El doctorado combinó tres contribuciones. En primer lugar, se desarrolló un método para cultivar el norovirus humano utilizando vellosidades intestinales aisladas de ratón como modelo celular que exhibe la diversidad completa de células epiteliales intestinales. Se probaron varias concentraciones de tripsina para activar norovirus; el método se aplicó a muestras fecales y ambientales. En segundo lugar, se evidenció la contaminación de *E. coli* y patógenos entéricos humanos en el agua (lixiviados de vertedero, agua de escorrentía, río, agua de riego, agua potable), vegetales de hoja que se comen crudos (acelgas) y aire (sobre un vertedero sanitario, así como en áreas rurales y urbanas) en la región de Bogotá. En tercer lugar, se evaluó el impacto de los contextos socioeconómicos y las prácticas individuales (alimentación, higiene y salud) frente a los casos de gastroenteritis aguda a partir de encuestas realizadas en una localidad de Bogotá y analizadas mediante diversas herramientas (análisis de componentes principales, modelización...).

Con este doctorado, hemos demostrado que las vellosidades intestinales aisladas de ratón permiten la infección y la replicación del norovirus humano. El virus debe activarse con tripsina y tiene un ciclo replicativo promedio de 10 h. Las vellosidades son eficaces para obtener abundante material biológico y son ideales para estudiar la actividad biológica de los norovirus o para generar anticuerpos. Ellas permitieron ver norovirus infecciosos no detectados por método molecular en varias heces y muestras ambientales; casi todas las muestras positivas por método molecular o inmunodot-blot contenían norovirus infecciosos.

A nivel regional, los vertidos de aguas residuales en los ríos Bogotá y Balsillas y en el humedal Tres Esquinas contaminan la red de riego La Ramada en el noroeste de Bogotá con *E. coli* y potencialmente con patógenos entéricos humanos. Las acelgas recolectadas en esta área resultaron muy contaminadas, a diferencia de otras áreas de cultivo. Su contaminación evolucionó desde la producción hasta su compra en las tiendas cercanas, los lavados aumentaron o disminuyeron su contaminación y la manipulación en los puestos de comercio aumentaron la contaminación. El aire a menudo estaba contaminado con *E. coli* y *Shigella* spp., sin poder atribuir al relleno sanitario *Doña Juana* un rol particular. A su vez la presencia de *Shigella* spp. se observó en paralelo en más de la mitad de las deposiciones de personas con diarrea. Las encuestas demostraron que la frecuencia anual de gastroenteritis aguda disminuye respecto al aumento en edad; parecía menos común en hogares con personas mayores, posiblemente debido a prácticas de higiene alimentaria más estrictas. Un modelo de transmisión de gastroenteritis aguda que distinguió la contaminación fuera de los hogares y las contaminaciones entre personas dentro de los mismos hogares no mostró diferencias significativas entre vecindarios. El instrumento utilizado para simular experimentos numéricos, sugiere trabajar en un número mucho mayor de encuestas.

Palabras clave : gastroenteritis aguda, patógenos entéricos, virus, bacterias, protozoos, transmisión fecal-oral, transmisión indirecta, contaminación del agua, contaminación por vegetales de hoja, contaminación del aire, vertedero, horticultura, riego, prácticas alimentarias, prácticas de higiene, prácticas de salud, condiciones socioeconómicas, seguimiento experimental, modelado, cultivo de norovirus humanos.



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Chapter I:

General Introduction

Human acute gastroenteritis (AGEs) affects a large proportion of the world population each year: e.g. approximately 33% of the French population (Van Cauteren et al., 2012), 55% of the US population (Lively et al., 2018), and probably more in Colombia, Brazil, Peru and Mexico in view of the data of Troeger et al. (2017) concerning only acute diarrhea, although data for France and the US are surprisingly low in this last work. They are responsible for morbidity, mortality and significant health costs (Troeger et al., 2018; Hoffman et al., 2015). In Colombia, Brazil, Peru and Mexico, the annual mortality of children <5 resulting from acute diarrhea would be around 1 in 1000 (Troeger et al., 2017; Ministerio de Salud y Protección Social, 2019), that is to say around 10 times more than in France or in the US (Troeger et al., 2017). Health costs vary not only with their aetiology (Hoffman et al., 2015), but also with age, the state of health and access to care of infected persons (Atchison et al., 2016; Ruiz-Rodríguez et al., 2012). The total costs (cost of treatment and value of time lost due to illness) for each case of foodborne AGE caused by *E. coli* O157:H7, non-typhi *Salmonella* spp., *Campylobacter* spp., *Shigella* spp. and noroviruses are estimated at US \$ 4,298, US \$ 3,568, US \$ 2,283, US \$ 1,041 and US \$ 413, respectively (Hoffman et al., 2015). By assigning the cost of a foodborne norovirus AGE case to all AGEs cases, the annual cost for France would exceed US \$ 8 billion (or more than €7 billion). The gains associated with a reduction in direct and indirect transmission of AGEs justify (and could finance!) research efforts, especially since there is a willingness to pay to avoid the disease, limit the pain and suffering of sick people and other people beyond the formalized costs (Hoffman et al., 2015).

Much knowledge already exists on AGEs. They can be caused by a wide variety of human enteric pathogens (viruses, bacteria, unicellular parasites) (Hoffman et al., 2015; WHO, 2015), or even by chemical compounds (Rather et al., 2017). Viruses have been increasingly recognized as an important cause of AGEs, particularly noroviruses (Glass et al., 2009) since the existence of rotavirus vaccines (López-Medina et al., 2018). AGEs are often transmitted from person to person – we speak of faecal-oral transmission – but they can also be transmitted through food (WHO, 2015), drinking water (WHO, 2015) and recreational waters (Leonard et al., 2015; Prez et al., 2015), inert surfaces (Stobnicka et al., 2018), or even air, at least indoors (Nazaroff, 2011). The relative importance of the different transmission routes depends on the pathogens (WHO, 2015), and in particular their capacity to cause zoonoses (Whitfield et al., 2017) and/or to survive in the environment (Gerba, 2007; Ives, 2007), or even to infect plants for a few (Jo et al., 2019)), beyond their simple surface contamination (Pérez-Rodríguez et al., 2019) or internalized contamination (DiCaprio et al.,

2015) without development of pathogens. The relative importance of the different transmission routes also depends on individual and collective food, hygiene and health practices (Lee et al., 2017), these practices themselves being linked to environmental, cultural and regulatory contexts resulting in differences between world regions (WHO, 2015). At the origin of the contamination, contaminated excrement or vomit, the fate of which depends in particular on the treatment of wastewater (World Water Assessment Program, 2017) and drinking water (WHO, 2011), on the hygiene of contaminated people (including hand washing (Lee et al., 2017)), as well as food packaging, storage and preparation practices (Sapers and Doyle, 2014).

But many methodological, scientific and finalized questions remain unanswered until present. Regarding methodologies, we do not know how to measure the infectivity or virulence of certain pathogens such as that of human noroviruses despite culture tests (Oka et al., 2018) and proposals to assess the integrity of viruses by molecular method (Lee et al., 2018). There is also a lack of methods to monitor in real time the contamination of water by pathogens, as the monitoring of faecal indicators is insufficient (Khan and Gupta, 2020). And there is a lack of non-destructive methods to accurately describe the fate of pathogens in three-dimensional environments such as soil, plants, and shellfishes. At the scientific level, knowledge relating to the inactivation of pathogens (for example viruses in the air (Girardin, 2015)) and/or their mutations in the environment (for example that of noroviruses (De Rougemont, 2018; Miyoshi et al., 2015)) are generally very insufficient understood for their integration into reliable quantitative microbial risk assessments (QMRA) (Membré and Guillou, 2016). And there are still many questions about the fate of human enteric human pathogens in different compartments of the environment. At a more finalized level, it seems essential to develop systemic approaches combining epidemic transmission of diseases, environmental fate of pathogens, human and social sciences (individual and collective practices, communication, health economics) to obtain models that are more integrative than the current models of epidemic transmission (Gaythorpe et al., 2018) and that enable more realistic quantitative microbial risk assessments.

In view of AGE risks, the Colombian context is specific from several points of view. Regarding water management, less than half of Colombian municipalities had wastewater treatment plants in 2016 (Gobierno de Colombia, 2018), of which about 17% carried out only primary treatment corresponding to about 43% of Colombian wastewater (Rodríguez Miranda et al., 2016); in Bogotá until now, only one wastewater treatment plant, *Salitre*, still does only primary treatment to treat about a third of the city's wastewater (the commissioning of

secondary treatment and tertiary treatment is planned for September 2021), while the rest of wastewater is discharged raw into the *Bogotá* River and its tributaries, the *Tunjuelito* and *Fucha* rivers, which cross the city (this water should be treated by a future wastewater treatment plant, *Canoas*, under design). Regarding food, the consumption of vegetables and fruits of the inhabitants of Bogotá is largely limited to a handful of relatively cheap products (Guarín, 2013) which may be irrigated by fresh surface water heavily contaminated with faecal indicators (Heliodoro and Bustos-López, 2018) and human enteric pathogens (FAO, 2017); however, the consumption of vegetables and fruits is quantitatively much lower than that of neighbouring countries, especially Venezuela and Mexico (Guarín, 2013). Finally, the daily disposal of about 6800 tons of waste (Molano-Camargo, 2019) with the entering of more than 680 camions with garbages (UAESP, 2020), waiting to discharge between 2.5 to 3.5 min into the landfill (Defensoría del Pueblo, 2004) and the disposal in landfill – without being covered by soil or clay for at least a few days (see **Chapter III**) – of soiled toilet paper and diapers for babies and adults induces potential risks of aerosolization of pathogenic agents (Girardin et al. al., 2016).

Also, the general objective of this PhD was to evaluate the flows of some human enteric pathogens in the environment near *Bogotá* and to try to relate these flows to the population health. This objective will be will be described more precisely after a review of the state of the art (**Chapter II**). A challenge for this PhD was linked to the very broad scope of the subject with work on different human enteric pathogens (viruses, bacteria, unicellular parasites) and on faecal indicators, with the study of their transfer by different vectors (food, water, air), and with an attempt to take into account individual practices (food, hygiene and health). The PhD was supervised by two complementary research units: in France, the *Unité Mixte de Recherche* (UMR INRAE & Avignon Université) *Environnement Méditerranéen et Modélisation des Agro-Hydrosystèmes* (EMMAH) in Avignon mainly providing its expertise on the fate of pathogens in environment, and in Colombia the *Laboratorio de Biología Molecular de Virus* of the *Universidad Nacional de Colombia* (*Facultad de Medicina, Departamento de Ciencias Fisiológicas, Unidad de Bioquímica*) in Bogotá providing mainly its expertise in virology and molecular biology. The work was supported by the Colombian COLFUTURO foundation for the PhD student's trips and stays in France, by INRAE for one-off salary support, and by the Nestle Foundation for the funding of experiments (small equipment, consumables and trips around *Bogotá*).

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Chapter II:

Bibliographic Review

I. Introduction

Acute gastroenteritis in humans (AGEs) affect a large proportion of the world population each year: approximately 33% of the French population (Van Cauteren et al., 2012), 55% of the population of the USA (Lively et al., 2018), and probably more in Colombia, Brazil, Peru and Mexico in view of the data of Troeger et al. (2017) concerning only acute diarrhea, although data for France and the US are surprisingly low in this last work. They are responsible for morbidity, mortality and significant health costs (Troeger et al., 2018; Hoffman et al., 2015). In Colombia, Brazil, Peru and Mexico, the annual mortality of children <5 resulting from acute diarrhea would be around 1 in 1000 (Troeger et al., 2017; Ministerio de Salud y Protección Social, 2019), that is to say around 10 times more than in France or in the US (Troeger et al., 2017). Health costs vary not only with their aetiology (Hoffman et al., 2015), but also with age, health status and access to care of infected persons (Atchison et al., 2016; Ruiz-Rodríguez et al., 2012). Upstream, they are affected by individual food, hygiene and health practices (Fernández and Gómez, 2010). Pathogens causing AGEs include viruses, bacteria, and unicellular parasites (Dennehy, 2019).

Although faecal-oral – or direct person-to-person transmission – often predominates in AGE transmissions, indirect transmissions are often important *via* food (Lively et al., 2018; Soto Varela et al., 2016; WHO, 2015), water (de Deus et al., 2019; WHO, 2015), air (Courault et al., 2017) – at least inside buildings (Nazaroff, 2011; Marks et al., 2003) –, and inert contaminated surfaces (Gerba and Kennedy, 2007). Indirect transmissions vary with pathogens (WHO, 2015) depending on their ability to survive or remain infectious in the environment (Rodríguez et al., 2009; Sedmak et al., 2003) and the existence of animal reservoirs (O'Brien, 2006). For all these reasons, indirect transmissions of AGEs vary by region (WHO, 2015). For the 'AMR B' geographical area, which includes most of the countries of Latin America (including Colombia), direct transmissions represent approximately 72%, 15%, 51% and 35% of transmissions of enteric norovirus, non-typhoidal *Salmonella* spp., *Shigella* spp. and *Cryptosporidium* spp., respectively (WHO, 2015). For the same geographical area, transmissions by food represent about 14%, 49%, 14% and 11%, while transmissions by water represent about 6%, 9%, 27% and 26% of transmissions of the same pathogens, respectively (WHO, 2015).

We can thus reduce the various transmission routes – direct and indirect – through public policies aimed at improving information for people, guaranteeing the quality of water and

food consumed raw, and facilitating access to care. Their implementation can combine very diverse means: communication, regulations and standards, incentives (financial or other), etc. In Colombia, the regulations already allow sanitary surveillance of food (*Decreto* 3075 of 1997 in particular) and drinking water (*Decreto* 1575 of 2007 in particular). In 2006, the World Health Organization proposed, in the third edition of its Guidelines for the Safe Use of Wastewater, Excreta and Gray Water in Agriculture and Aquaculture (WHO, 2006), a flexible approach to reduce health risks to realistic levels for given local contexts; given the current ranges of concentrations of human enteric pathogens in domestic wastewater and when this water is used for irrigation, these recommendations often amount to achieving a reduction in the content of certain pathogens between around 4 and 6 log₁₀, but without necessarily obtain it by the treatment of wastewater only, knowing that irrigation techniques, washing after harvest and waiting times between harvest and consumption of products could also contribute to this reduction.

In this bibliographical review, we present some generalities on AGEs (definition, aetiology, clinical symptoms, epidemic transmission). We then discuss more comprehensively with the processes underlying the indirect transmission of enteric pathogens to humans. We finish by listing the main experimental methodologies allowing to trap or elute these pathogens from different matrices (air, water, food), then to concentrate and/or purify them, and finally to enumerate them. We briefly mentioned a few indicators of faecal contamination, the monitoring of them being likely to enrich the understanding of the processes involved in the fate of human enteric pathogens.

II. AGEs: medical signs, clinical symptoms, epidemiology, aetiology

II.1. Generalities

The term "gastroenteritis" comes from the Greek; it associates 'gastron' for stomach with 'enteron' for small intestine, to signify that it is an inflammation of the mucous membranes of the gastrointestinal tract (Al Jassas et al., 2018; Noguera et al., 2014; Chow et al., 2010; Costa et al., 2010; Webb and Starr, 2005).

The most common clinical symptoms resulting from AGEs are diarrhoea and/or vomiting (Pleşea et al., 2016; Noguera et al., 2014; Chow et al., 2010; Costa et al., 2010; Webb and Starr, 2005; Lopman et al., 2004; Rockx et al., 2002), often in addition to nausea, intestinal

cramps and fever (Pleșea et al., 2016; Fernández and Gómez, 2010; Glass et al., 2009; García, 2006). Diarrhoea can cause dehydration in people (Pleșea et al., 2016; Churgay and Aftab, 2012; Colletti et al., 2009), which can result in dry skin, headaches, sunken eyes, dizziness, confusion, and sometimes death (Hartling et al., 2006). AGEs are a frequent cause of morbidity and mortality for elderly people in retirement homes (Thouillot et al., 2012); Harris et al. (2008) estimated that 80 deaths of people >64 years are due to norovirus infections each year in England and Wales.

While gastroenteritis is defined as an inflammation of the mucous membranes of the gastrointestinal tract leading to diarrhoea and/or vomiting (Kazemian et al., 2015; Chau et al., 2015), some authors have clarified their definition. Thouillot et al. (2012) define AGEs as the sudden onset of at least two episodes of vomiting or two episodes of diarrhoea within a 24 h period. Similarly, the World Health Organization (WHO) considers that a person suffers from diarrhoea when there is simultaneously a decrease in the consistency of the stool and an increase in the frequency of evacuation with more than 3 stools in 24 h (WHO, 2005). And Webb and Starr (2005) defined as acute diarrhoea in breastfeeding babies the sudden increase in the quantity of stool fluid above a value of 10 mL of stool per kg of body weight per day.

The pathogens causing human AGEs include different viruses: noroviruses of genogroups I, II and IV (Zheng et al., 2006), type A rotaviruses mainly affecting very young children, certain enteroviruses (echovirus and coxsackievirus) (Sedmak et al., 2003), adenoviruses (Rojas et al., 2011), astroviruses (Bosch et al., 2014) Several bacteria also cause AGEs: enterohaemorrhagic *E. coli* (Nguyen and Sperandio, 2012), enterotoxigenic *E. coli* (Mentzer et al., 2014), *Shigella* spp., *Salmonella* spp., *Campylobacter* spp. (Elliot and Dalby-Payne, 2004) Finally, unicellular parasites can cause AGEs: *Cryptosporidium* spp. and *Giardia Lamblia* (Elliot and Dalby-Payne, 2004)

II.2. Aetiology, medical signs and clinical symptoms of AGEs

AGEs of viral aetiology appear to be preponderant (Dennehy, 2019; Fernández and Gómez, 2010; Glass et al., 2009; Elliot and Dalby-Payne, 2004). They could correspond to 70-80% of AGEs by simultaneously considering all age groups (Díaz et al., 2012; Marie-Cardine et al., 2002; Rockx et al., 2002). For children <5 years only, several authors have reported that 70% of AGEs are of viral aetiology (Chung et al., 2017; Elliott, 2007; Elliot and Dalby-Payne, 2004), 10-20% would be caused by bacterial aetiology (Elliot, 2007; Elliot and Dalby-Payne, 2004), and less than 10% would be caused by unicellular parasites (Elliott, 2007). AGEs of

viral aetiology are often caused by noroviruses in the World (Glass et al., 2009; Rockx et al., 2002), in Latin America (Da Silva Poló et al., 2016; Riveros and Ochoa, 2015) and in Colombia (Fernández et al., 2015), especially since the availability of rotavirus vaccines (López-Medina et al., 2018; Peláez-Carvajal et al., 2014; De la Hoz et al., 2010). In temperate climates, AGEs of viral aetiology are generally much more frequent during winter periods while, conversely, AGEs of bacterial aetiology seem a little more frequent in summer periods (Baumann et al., 2012). We sometimes speak of 'Winter Vomiting Disease' for norovirus AGEs (Gaythorpe et al., 2018). The same pathogens are found in other climates (WHO, 2015; Gutiérrez et al., 2006; Gutiérrez et al., 2005; Esona et al., 2003), with in Colombia for example more AGEs of viral aetiology during rainier periods and less clear variations of AGEs of bacterial aetiology with seasons (Gutiérrez et al., 2006; Gutiérrez et al., 2005).

While AGEs affect all age groups (Riveros and Ochoa, 2015; Glass et al., 2009; Rockx et al., 2002), the people most at risk are young children (<5 years), the elderly and immunocompromised people (Fernández and Gómez, 2010). Each year between 2000 and 2003, around 1.9 million children <5 years died worldwide following an AGE, explaining 18% of the mortality of this age group, just after pneumonia which explained 19% of mortality of the same age class (Bryce et al. 2005). Parry et al. (2013) doing a retrospective study over the years 1982 to 2006 showed that the mortality rate following infection with non-typhoidal *Salmonella* spp. increased with the age: 0%, 2%, 6.8%, 10.9% and 20% among 16-55 years old, 56-65 years old, 66-75 years old, 76-85 years old and >85 years, respectively. Finally, *Cryptosporidium* spp. are among the most frequent and serious opportunistic intestinal parasites of people with AIDS (Gholami et al., 2016; Ghorbanzadeh et al., 2012; Brink et al., 2002). Elimination of *Cryptosporidium* oocysts with the stool can continue indefinitely (De la Parte-Pérez et al., 2005), and the prevalence of cryptosporidiosis varies with age (Gholami et al., 2016; Ghorbanzadeh et al., 2012; Nahrevanian et al., 2008; Lindo et al., 1998), people of 30-39 years being the most affected (Gholami et al., 2016).

Clinical symptoms vary with the aetiology of AGEs (Tesson et al., 2019; Corcoran et al., 2014). AGEs of viral aetiology are very often brief – about 2 to 6 days – (Tesson et al., 2019; Chung et al., 2017; Rockx et al., 2002) unlike AGEs of bacterial aetiology, which the duration sometimes exceeds 1 week (Tesson et al., 2019; Chung et al., 2017) or AGEs of parasitic aetiology – around 6.5 days for an AGE caused by *Cryptosporidium* spp. (Chappell et al., 1999) –. AGEs of bacterial aetiology are more frequently accompanied by fever than AGEs of viral aetiology (Tesson et al., 2019; Uhnou et al., 1986). Vomiting symptoms are more frequent in AGEs of viral aetiology than in AGEs of bacterial aetiology (Tesson et al., 2019;

Uhnoo et al., 1986). Finally, the presence of mucus is more frequently observed in the stools of people suffering from AGEs of bacterial aetiology (Wiegering et al., 2011). But for the same aetiology, these symptoms vary with people, especially with their age (Díaz et al., 2012). For example, Rockx et al. (2002) reported that the duration of symptoms caused by Norwalk virus (norovirus GI.1) decreases with increasing age (6, 5 and 3 days in median values for children <1 year, 5-11 years and >12 years, respectively). But for AGEs of the same aetiology, the symptoms are also variable between infected people of the same age category (Tesson et al., 2019; Teunis et al., 2015; Miyoshi et al., 2015). Their consequences may vary from region to region, including Disability Adjusted Life Years (DALY) which may be higher in developing countries than in industrialized countries (WHO, 2006). To assess the viral aetiology or not of an AGE, several indicators have thus been proposed: the Fontana and Kaplan scores for a sick person (Kaplan et al., 1982; Fontana et al., 1987), the CART model for a cohort of sick people (Lively et al., 2018), and the proportion of cases of vomiting in a population infected with different pathogens (Tesson et al., 2019).

Beyond their direct effects, some AGEs can be followed by chronic sequelae. These sequelae include necrotizing enterocolitis in very young children <1 year (Neu and Walker, 2011; Fernández and Gómez, 2010) following infections by *Shigella* spp. (Grishin et al., 2013; Bello et al., 2003; Sirivichayakul and Thisyakorn, 1998), by *Salmonella* spp. and *E. coli* (Grishin et al., 2013; Fustiñana, 2011; Coates et al., 2005), as well as probably by rotaviruses (Fustiñana, 2011), adenoviruses, coronaviruses, echovirus 7 and astroviruses (Grishin et al., 2013). Chronic sequelae also include bacteraemia, i.e. occasional release of bacteria into the blood from an infectious hot-spot (Shahunja et al., 2015) giving rise to tremors and potentially fatal increases in temperature (Acheson and Hohmann, 2001). They affect about 5% of people with AGE caused by non-typhoidal *Salmonella* spp. (Acheson and Hohmann, 2001) and are more likely to occur in immune-deficient patients (Acheson and Hohmann, 2001) and/or severely underfed, clinically diagnosed sepsis or pneumonia (Shahunja et al., 2015). Other authors have reported fatal bacteraemia caused by *Shigella sonnei* infections in adults >62 years of age with cancer (Liu et al., 2009a). Chronic sequelae also include chronic diarrhoea of 12 months or more with persistent shedding of norovirus in people of all ages with cancer and/or lymphoid leukaemia (He et al., 2017; Capizzi et al., 2011), as well as than diarrhoea with prolonged dehydration caused by noroviruses and leading to stunted growth in children <5 years (Simon et al., 2006). Finally, exposure to *Cryptosporidium parvum* in healthy individuals can lead to transient infection ranging from asymptomatic to severe (Macarisin et al., 2010). In immunocompromised patients in the absence of effective drug

therapy, it can be fatal (Rossignol, 2009; Kortbeek, 2009; Fayer and Xiao, 2008). It can cause anaemia, stunted growth, and intellectual and cognitive retardation in children (Karshima, 2018).

II.3. Direct and indirect transmissions; the existence of zoonoses

While direct person-to-person (faecal-oral) transmission is often the main route of AGE transmission (Al Jassas et al., 2018; Pleşea et al., 2016; Noguera et al., 2014), AGEs can be transmitted indirectly *via* the environment: water (Rodríguez et al., 2009), food (Lively et al., 2018; Soto Varela et al., 2016), air (Masclaux et al., 2014) and inert contaminated surfaces (Abad et al., 1994). Among the factors affecting the transmission of pathogens, zoonoses exist for *Salmonella* spp. which also infect poultry (Afzal et al., 2015), for *Cryptosporidium* spp. which also infect cattle (Paul et al., 2008), and for *Shigella* spp. which not only infect humans and other primates (McLennan et al., 2018) but also fresh vegetables (Jo et al., 2019). Beyond that, the relative importances of direct transmissions and the various indirect transmission routes also depend on the regional context, particularly on everything relating to water management, food production and distribution, individual food, hygiene and health practices. For example, the proportions of direct and indirect transmission of pathogens differ between non-typhoidal *Salmonella* enterica, *Shigella* spp., Enteropathogenic *E. coli* and norovirus, but also, for these same pathogens, between the 'AMR A' sub-regions (including the Canada and the USA) and 'AMR B' (including most Latin American countries including Colombia) (Table 1).

III. Indirect transmissions of pathogens to people

Human enteric pathogens can be shed with vomiting and/or stool. Even if their concentrations are lower in vomiting than in stool (about 100 times lower for noroviruses (Makison-Booth, 2014; Atmar et al., 2008)), the risk of transmission by microdroplets in suspension during vomiting episodes may be higher than the risk of transmission through stool (Makison-Booth, 2014). Note that for some pathogens responsible for AGEs, some people can be infected without being sick. This is for example the case for noroviruses GII with about 30% asymptomatic carriers (Teunis et al., 2015) which shed the viruses in their stool at similar concentrations (Atmar et al., 2008; Teunis et al., 2015) or lower concentrations (Gallimore et

Sub-region	Food	Contact with animals	Human to human contact	Water	Soil	Other
Non-typhoidal <i>Salmonella enterica</i>						
AMR A	0.73 (0.38–0.91)	0.10 (0.00–0.39)	0.05 (0.00–0.28)	0.02 (0.00–0.22)	0.00 (0.00–0.09)	0.00 (0.00–0.05)
AMR B	0.49 (0.09–0.74)	0.19 (0.00–0.45)	0.15 (0.00–0.40)	0.09 (0.00–0.32)	0.01 (0.00–0.12)	0.02 (0.00–0.05)
<i>Shigella</i> spp.						
AMR A	0.12 (0.00–0.46)	na	0.69 (0.33–0.93)	0.10 (0.00–0.41)	0.00 (0.00–0.21)	0.00 (0.00–0.06)
AMR B	0.14 (0.00–0.52)	na	0.51 (0.10–0.81)	0.27 (0.03–0.61)	0.00 (0.00–0.18)	0.00 (0.00–0.06)
Enteropathogenic <i>E. coli</i>						
AMR A	0.72 (0.20–0.97)	0.00 (0.00–0.31)	0.11 (0.00–0.53)	0.00 (0.00–0.57)		0.00 (0.00–0.01)
AMR B	0.29 (0.01–0.62)	0.00 (0.00–0.34)	0.16 (0.00–0.50)	0.46 (0.12–0.76)		0.00 (0.00–0.01)
<i>Cryptosporidium</i> spp.						
AMR A	0.16 (0.01–0.44)	0.10 (0.01–0.42)	0.30 (0.03–0.64)	0.37 (0.08–0.72)		0.00 (0.00–0.09)
AMR B	0.11 (0.01–0.38)	0.20 (0.02–0.47)	0.35 (0.07–0.66)	0.26 (0.05–0.61)		0.00 (0.00–0.09)
Norovirus						
AMR A	0.23 (0.04–0.50)		0.50 (0.18–0.79)	0.22 (0.00–0.49)		0.00 (0.00–0.22)
AMR B	0.14 (0.00–0.42)		0.72 (0.36–0.90)	0.06 (0.00–0.40)		0.04 (0.00–0.24)

Table 1: Relative proportions of the different transmission routes of some pathogens responsible for human AGEs (taken from WHO (2015)). Numbers in parentheses represent 95% confidence intervals for these proportions.

al., 2004; Chikhi-Brachet et al., 2002) than virus concentrations in the stools of symptomatic infected persons. Pathogens can thus be released with excrements into wastewater (Tesson et al., 2019), and end up diluted in water bodies (rivers, aquifers, lakes, marshes) if they have not been eliminated by wastewater treatment (Prevost et al., 2015). Their subsequent fate combines transfer (Tesson et al., 2019), inactivation (loss of infectivity or mortality) (Rodríguez et al., 2009), immobilization on solids (Abad et al., 1994) and/or filtration by living organisms (seafood, etc.) (Pavoni et al., 2013). The risk of indirect human infection then depends in part on water use: drinking water (Haramoto et al., 2004), irrigation water (Heliodoro and Bustos-López, 2018) and recreational water (Sanborn and Takaro, 2013). In the case of irrigations with contaminated water, the contamination may concern the external surfaces of agricultural products (Hirneisen and Kniel, 2013; Wheeler et al., 2005), or even the interior of organs eaten raw after internalization at the level of roots and migration into the plant with the flow of sap (Yang et al., 2018; DiCaprio et al., 2015) or sometimes at the level

of stomata or wounds of plant organs (Potnis et al., 2015; Berger et al., 2015; Berger et al., 2010; Wang et al., 2003). But water is not the exclusive vector of indirect contamination: alongside airborne transmissions initiated by vomiting, certain pathogens deposited on solids can be aerosolized (Girardin et al, 2016); and poor hygiene can result in contamination of fomites (Pickering et al., 2012) and food during postharvest handling (Girmay et al., 2020).

III.1. Water transmission

Raw domestic wastewater can have high concentrations of human enteric pathogens. The enteric virus concentrations can range from 10 to 10^{+7} GC.mL⁻¹ (for Genomic Copies per mL) (Table 2). Likewise, human pathogenic enteric bacteria are present in raw wastewater at concentrations ranging from approximately 1 to 10^{+4} GC.mL⁻¹ or cfu.mL⁻¹ (for colony forming unit per mL) (Table 3). Finally, numerous studies have shown the presence of unicellular parasites in raw wastewater, including *Cryptosporidium* spp. and *Giardia* (Table 4), but studies also noticed the importance of *Entamoeba* spp. in Colombia (Fonseca et al., 2016), in agreement with Entamoeba detection in human stools (Consuelo López et al., 2015).

Virus	Concentration (GC.mL ⁻¹)	Reference
Enterovirus (Poliovirus, Coxsackievirus A, Coxsackievirus B, Echovirus)	$1.0 \cdot 10^{+2} - 2.4 \cdot 10^{+6}$	Schmitz et al. (2016) Kitajima et al. (2014) La Rosa et al. (2010) González et al., 2006
Adenovirus	$1.0 \cdot 10^{+0} - 1.0 \cdot 10^{+6}$	La Rosa et al. (2010) Hewitt et al. (2011)
Norovirus (GI et GII)	$4.8 \cdot 10^{+5} - 9.3 \cdot 10^{+6}$	Teixeira et al. (2020) La Rosa et al. (2010)
Rotavirus	$2.0 \cdot 10^{+0} - 7.2 \cdot 10^{+2}$	Kobayashi. (2017) Paranychianakis et al. (2010) Chigor and Okoh (2012)
Human astrovirus	$8.1 \cdot 10^{+2} - 2.1 \cdot 10^{+7}$	Kobayashi. (2017) Le Cann et al. (2004)
Hepatitis A virus		Báez et al., 2016

Table 2: Enteric virus concentrations measured in raw wastewater (adapted from Sidhu and Toze (2009)). Báez et al. (2016) and González et al. (2006) detected viruses in Colombia wastewaters without quantifying them.

The treatment of raw domestic wastewater contributes to reducing the concentrations of human enteric pathogens in such water. Different types of treatments exist. Pre-treatment or primary treatment can combine all or part of the 'screening-sand removal-oil removal' chain

Country	<i>E. coli</i> cfu.mL ⁻¹	<i>Salmonella spp.</i> cfu.mL ⁻¹ / MPN.mL ⁻¹	<i>Shigella spp.</i> GC.mL ⁻¹	References
Tanzania	-	40 à < 400	-	Mhongole et al. (2017)
China	-	-	10 10 ⁺¹	Zhou et al. (2015)
Brazil	1.0 10 ⁺³	1.0 10 ⁺⁴	-	Viancelli et al. (2015)
Japan	-	-	1.3 10 ⁺⁰	Ishii et al. (2014)
Colombia	2.0 10 ⁺⁴	-	-	Venegas et al. (2014)
USA	3.2 10 ⁺³	-	-	Nelson et al. (2008)
Japan	1.0 10 ⁺⁵	-	-	Haramoto et al. (2006)
Spain	-	2.7 10 ⁺²	-	Howard et al. (2004)
Spain	1.2 10 ⁺⁵	1.0 10 ⁺³	-	Torrella et al. (2003)

Table 3: Concentrations of human enteric bacteria in raw wastewater expressed as GC.mL⁻¹, cfu.mL⁻¹ or MPN.mL⁻¹ (for Most Probable Number per mL).

(Tesson, 2018; Sonune and Ghate, 2004); it allows the removal of solids and part of the grease present in wastewater. When it exists, secondary treatment – very generally by "activated sludge" – makes it possible to achieve most of the reduction in organic matter, or even in mineral nitrogen and phosphates by adjusting the aeration of the environment (Sonune and Ghate, 2004). It is very generally followed by clarification of the water with flocculation of the microflora constituting the "activated sludge" and possibly precipitation of phosphates (Haydar et al., 2016; Sonune and Ghate, 2004). In some cases, the "activated sludge" treatment is integrated in a membrane bioreactor (MBR) allowing ultrafiltration of the treated

Countrys	<i>Cryptosporidium spp.</i> (oocystes.L ⁻¹)	<i>Giardia</i> (kystes.L ⁻¹)	Reference
Colombia	233 – 267	-	Alarcón et al. 2005
USA	0.1 – 1 000	1 – 10 000	Harwood et al. (2005)
USA	0.61 – 120	1 – 130	Rose et al. (1996)
China	33 – 600	130 – 3 600	Fu et al. (2010)
Israel	4 – 125	10 – 12	Taran-Benshoshan et al. (2015)
Israel	8.3 – 8.5	5–27.3	Zuckerman et al. (1997)
Sweden	20	250 – 12 500	Ottoson et al. (2006)
Canada	1 – 560	100 – 9 200	Payment et al. (2001)
United-kingdom	10 – 170	10 – 13 600	Bukhari et al. (1997)
Spain	40 – 340	-	Montemayor et al. (2005)
Italy	2.5 – 40	2,100 – 42 000	Caccio et al. (2003)
Norway	100 – 1 100	100 – 13 600	Robertson et al. (2006)
Malaysia	1 – 80	18 – 8 480	Lim et al. (2007)

Table 4: Concentrations of *Cryptosporidium spp.* and *Giardia* in raw wastewater reported by various authors. Table adapted from Fu et al. (2010).

water (Sahar et al., 2011); it is considered that MBR combines secondary and tertiary treatments. Alternative treatments exist, in particular lagooning which can replace treatment by "activated sludge" for small towns (Tesson, 2018) or succeed it as a tertiary treatment (Malovanyy et al., 2018), and chemical treatment in tourist places where the population varies too much during the year to be able to maintain "activated sludge" (Duchène, 2005). When they exist, tertiary treatments are intended to reduce the levels of pathogens and/or micropollutants. They are based on chemical oxidations by hypochlorite, ozone (Gomes et al., 2019) or peracetic acid (Shah et al., 2015), on UV-C irradiations (~254 nm) attacking DNA and RNA, or on various filtrations after possibly flocculation and/or chemical precipitation (Chevremont et al., 2012; Sonune and Ghate, 2004). In the extreme, reverse osmosis, considered as quaternary treatment, removes all chemical compounds from water (Corral et al., 2014); Classically used to desalinate seawater and brackish water, it also reduces the salinity of certain wastewaters before they are reused in agricultural irrigation (Tesson, 2018). Pathogen removals vary with the pathogens and the treatments. Concerning enteric viruses, pretreatments and secondary treatments with "activated sludge" generally allow a removal of less than 1 log₁₀ (La Rosa et al., 2012) to 3-4 log₁₀ (Campos et al., 2016), with strong dependence on viruses (da Silva et al., 2007), tertiary treatments allowing an additional reduction (Verbyla and Mihelcic, 2015). For examples: for the 5 purification stations around Rome (Italy) with chlorine disinfection, La Rosa et al. (2010) observed reductions of only 0.7 and 0.4 log₁₀ for the noroviruses GI and GII, respectively; for the "Les Trois Rivières" wastewater treatment plant near Clermont-Ferrand (France) with the same type of pre-treatment and secondary treatment but without chlorine disinfection, Tesson et al. (2019) noted average year-scale removals of about 2.4 and 1.9 log₁₀ for noroviruses GI and GII, respectively. The use of MBR allows a higher removal of noroviruses GII with log₁₀ values of 4.7 (Simmons and Xagorarakis, 2011), 3.35 (Sano et al., 2016), 2.4 (Campos et al., 2016) and 2.3 (Purnell et al., 2016), unlike activated sludge treatments without MBR, for which are reported removals of 1.4 (Sano et al., 2016), 0.9 (Flannery et al., 2012) and 0.4 log₁₀ (La Rosa et al., 2010). Concerning human pathogenic enteric bacteria, pre-treatments and secondary "activated sludge" treatments generally allow a removal ranging from 4 to 7 log₁₀ for *E. coli* (Wen et al., 2009), but the use of MBR can drastically increase the removal in pathogens and bacteria indicative of faecal contamination, or even retain them entirely in filtration (Hai et al., 2014; Ueda and Horan, 2000). Tertiary treatments allow an additional removal, sand filtration (Hoslett et al., 2018; Fu et al., 2010; Jiménez-Cisneros et al., 2001) being less efficient than ultrafiltration (Hai et al., 2014) but it certainly depends on the flocculation/coagulation which

may precede it (Hoslett et al., 2018); removals comparable to those obtained by ultrafiltration can be obtained by UV-C irradiation (Sánchez-Montes et al., 2020) or by chemical oxidations (Jiménez-Cisneros et al., 2001). For example: Surampalli et al. (1994) reported removals in *Salmonella* spp. of about 2 log₁₀ for pre-treatment followed by secondary treatment with activated sludge in Jefferson, Rochepport, New Bloomfield, Osage Beach, Blue Springs (Mo, USA) and in Fulton (NY, USA); these values are close to the removals in *E. coli* obtained under similar conditions by Marín et al. (2015) in Navarre (Spain) and by La Rosa et al. (2010) in Rome (Italy); Jiménez-Cisneros et al. (2001) noted removals of 4.5 log₁₀ in total coliforms and in *Salmonella* spp. by peracetic acid oxidation after secondary treatment in Mexico City (Mexico); Kadam et al. (2008) reported a 1.9 log₁₀ reduction in *E. coli* O157:H7 concentrations using planted soil filters in two factories east of Mumbai (India); and Hench et al. (2003) show a *Shigella* removal of 2 and 1.4 log₁₀ in a plant in Morgantown (Va., USA) using constructed wetlands vegetated or not, respectively. Concerning unicellular parasites, the pre-treatments and secondary treatments with “activated sludge” generally allow a removal ranging from 1 to 4 log₁₀ and from 1 to 3 log₁₀ for *Cryptosporidium* spp. and for *Giardia* spp., respectively (Wen et al., 2009), but the use of MBR can drastically increase their removal, or even retain them entirely during filtration (Hai et al. 2014; Ottoson et al., 2006). Among tertiary treatments, the effectiveness of chlorine disinfection on *Cryptosporidium parvum* removal depends on the dose and exposure time (Rennecker et al., 2000; Korich et al., 1990). Similarly, the efficiency of the filtration on the depletion of oocysts of *Cryptosporidium* spp. simultaneously depends on the filter and the flocculation processes implemented before (Nasser et al., 2016; Fu et al., 2010; Hirata and Hashimoto 1998). And UV irradiation can be very effective in removing *Cryptosporidium* spp. oocysts. (Nasser et al., 2016; Morita et al., 2002). For example: Montemayor et al. (2005) reported a removal in infectious *Cryptosporidium* oocysts of about 2.7 log₁₀ for a treatment ultimately including sand filtration, chlorination and UV irradiation in Barcelona (Spain); Nasser et al. (2016) considered that treatment with “activated sludge” followed by sand filtration is ineffective in killing *Cryptosporidium* spp., But Dugan et al. (2001) noted that optimal coagulation followed by filtration allowed a removal of more than 4 log₁₀ in oocyst concentrations of *Cryptosporidium* spp.; and Hirata and Hashimoto (1998) had already noted that microfiltration and ultrafiltration allow a removal of *Cryptosporidium* oocysts of more than 7 log₁₀; aerobic and anaerobic lagoons allow removals between 2-5 log₁₀ (Nasser et al., 2016; Reinoso et al., 2008). Thus, the removals in human enteric pathogens are generally very incomplete in wastewater treatment plants with, however, more risk of virus release than

pathogenic bacteria or unicellular parasites (Tesson, 2018; La Rosa et al. al., 2010), bacteria and unicellular parasites being more easily retained in sludge from wastewater treatment plants and/or by filtration (Rodríguez-Mozaz et al., 2015), even if these results may be discussed. This can result in water unsuitable for unrestricted agricultural irrigation (Banach and Van der Fels-klerx, 2020; Uyttendaele et al., 2015; Cirelli et al., 2008).

Worldwide, it is estimated that around 80% of domestic wastewater is still discharged into the environment without treatment (World Water Assessment Program, 2017). The levels of equipment in well-equipped countries often result from recent developments, such as in Europe following the Water Framework Directive (European Parliament, 2000). Currently in Colombia, around 19%, 59% and <1% of wastewater, at least partly of domestic origin, would be released into the environment after a pre-treatment (or primary treatment), a secondary treatment or a tertiary treatment, respectively (Superintendencia de Servicios Públicos, 2014). The situation can sometimes be difficult to assess. If the existence of a sewer network is a prerequisite for collective sanitation, sewer network can simultaneously collect rainwater (case of a unitary network) or not (case of separate networks for networks collecting wastewater and rainwater, respectively), sometimes with the juxtaposition of a unitary network and a separate network in the old and recent neighbourhoods of the same city, respectively. In the case of a unitary network, part of the wastewater (diluted by rainwater) can be diverted through a bypass and be discharged into the environment without treatment during periods of heavy rains (Tesson, 2018; Tesson and Renault, 2017; Lipp et al., 2001), so as not to damage the activated sludge.

Most wastewater treatment plants discharge their treated wastewater directly into surface water bodies, where its dilution reduces pollution (Tesson and Renault, 2017). This can result in contamination of the surface water bodies receiving raw or treated wastewater with human enteric pathogens: rivers (Bhasin et al., 2020; Tesson et al., 2019; Vermeulen et al., 2019; Partyka et al., 2018; Prevost et al., 2015; Botes et al., 2013; Olivas-Enriquez et al., 2011; Girones et al., 2010; Ahmed et al., 2009; Liu et al., 2009b), karstic or non-karstic aquifers (Mahagamage, et al., 2019; Haramoto 2018; Olalemi and Auda, 2018; Balderrama-Carmona et al., 2017; Balthazard-Accou et al., 2017; De Giglio et al., 2017; Hench et al., 2003; Abbaszadegan et al., 2003; Fout et al., 2003), seas (Betancourt et al., 2014; Pavoni et al., 2013; Rodrigues et al., 2011; Rose et al. , 2006; Katayama et al., 2002; Rozen and Belkin, 2001; Fayer et al., 1998), or even surface reservoirs for agricultural irrigation (Partyka et al., 2018). The fate of human enteric pathogens in each of the water compartments (river, lake, marsh, aquifer, sea, etc.) combines transfer, immobilization/remobilization, multiplication

and/or inactivation/mortality. Dilution and transport are closely associated with the water flows. The immobilizations/remobilizations and inactivation and/or mortality of pathogens depend on a multitude of local parameters such as temperature (Murphy, 2017; Bertrand et al., 2012; Wetz et al., 2004), radiation – UV in particular – (Murphy, 2017), suspended particles (sediments, colloids, etc.) and physicochemical characteristics (salinity, pH, dissolved oxygen and redox state of the medium, organic matter, biology ...) (Tesson et al., 2018; Girardin et al., 2016; Kotwal and Cannon, 2014; Gerba, 2007; John and Rose, 2005; Lipp et al., 2001; Šolić and Krstuvolic 1992). In certain cases, the immobilization of a pathogen can protect it or, conversely, promote its inactivation: this is particularly the case for certain viruses (Nagvenkar and Ramaiah, 2009; Lipp et al., 2001; Gerba and McLeod 1976) which can be protected by biofilms (Skraber et al., 2009), or whose inactivation can be slowed down (Schaub and Sagik, 1975; Gerba, 1999) or accelerated following their adsorption to the surface of certain minerals, in particular metal oxides (Zhuang and Jin, 2008; Murray and Laband, 1979). In water, certain pathogens can at most survive and retain their infectivity, such as enteric viruses (Rzeżutka and Cook, 2004), *Shigella* spp. (Jo et al., 2019), and *Cryptosporidium* spp. as oocysts (Widmer et al., 2020)); others such as *E. coli* can multiply under certain conditions (Aruscavage et al., 2006). Temperature affects the fate of pathogens: an increase in temperature promotes the inactivation of human enteric viruses (Dalziel et al., 2016; Bertrand et al., 2012; Bae and Schwab, 2008; Wetz et al., 2004) because they increase the risk of denaturing the capsids and genomes of viruses (Tesson et al., 2018). Bacteria are sometimes more resistant to high temperatures (Baumann et al., 2012).

Pathogens found in water compartments (rivers, aquifers, etc.) may eventually be found in drinking water (Karim et al., 2009; Haramoto et al., 2004) and/or recreational waters (Prez et al., 2015), and irrigation water (Heliodoro and Bustos-López, 2018). Regarding the contamination of irrigation water with pathogens, El-Senousy et al. (2013) showed that 32% of samples of water of natural origin used for this activity and positive for the norovirus GI, contained mean concentrations of 10^{+2} CG.L⁻¹ of the virus, while the concentrations in *Salmonella* spp., *Cryptosporidium* spp. and total coliforms in the same types of water had mean values of $2.39 \cdot 10^{+6}$ cfu.(100 mL)⁻¹, $2.6\text{-}3.2 \cdot 10^{+2}$ oocysts.(100 mL)⁻¹ and $7.6 \cdot 10^{+10}$ cfu.(100 mL)⁻¹, respectively (Henao-Herreño et al., 2017; Pachepsky et al., 2011; Kay et al., 2008; Rose et al., 2001). The average concentrations of *E. coli*, an indicator of faecal contamination, were of the order of $2.36 \cdot 10^{+2}$ cfu.(100 mL)⁻¹ in this type of water (Antaki et al., 2016).

III.2. Food transmissions (leafy-vegetables, fruits/nuts, shellfishes ...)

Several human enteric pathogens cause zoonoses by infecting animals (Whitfield et al., 2017; Eng et al., 2015; Schmutz et al., 2016; Painter et al., 2015; Markland et al., 2013). Host animals then become reservoirs for these pathogens, which they can release with their excrements into the environment (Besser et al., 1997; Graczyk et al., 1997) or transmit to humans through their meat and/or by-products (eggs, milk, cheese, etc.) (Heredia and García, 2018; Sonnier et al., 2018; Soto-Varela et al., 2018; Antunes et al., 2016; Boonmar et al., 2013). Among the pathogens concerned, non-typhoidal *Salmonella* spp. (i.e. other than *Salmonella typhi* and *Salmonella paratyphi* which infect only humans) are usually detected in poultry (chicken, turkey, etc.) (Antunes et al., 2016; Sanchez et al., 2002), in pork (including ham) and in eggs (Soto Varela et al., 2016; Kramarenko et al., 2014; Favier et al., 2013); beyond that, reptiles are healthy carriers of *Salmonella* spp. and, as "new pets", can cause severe infections in infants (Wen et al., 2017; Pflieger et al., 2003). Likewise, some strains of pathogenic *E. coli* are reported in meats (beef, pork, chicken), sea food and fish (Soto Varela et al., 2016; Balière et al., 2015; Bandyopadhyay et al., 2012; Canizalez-Roman et al., 2013). They include enteropathogenic *E. coli*, enteroaggregative *E. coli*, enterotoxigenic *E. coli*, diffusely adherent *E. coli*, enteroinvasive *E. coli*, and Vero toxin-producing/Shiga toxin-producing *E. coli* including enterohaemorrhagic *E. coli* (Canizalez-Roman et al., 2013; Kaper et al., 2004). *E. coli* O157:H7 belong to the latest category; their main habitat is inside the intestines of cattle (Moxley, 2004) and they are the source of poisoning often transmitted by minced meat (Tavakoli et al., 2008) – sometimes referred to as “hamburger disease” (Vallance et al., 2003) –. *Cryptosporidium* also infect cattle – especially younger ones – (Avendaño et al., 2010; Broglia et al., 2008) as well as other domestic and wild animals (dogs, cats, chickens, horses, goats, sheep, pigs, birds, rodents ...) (Schaefer et al., 2020; Johnson et al., 2008; Ryan et al., 2005). Finally, a few human enteric viruses cause zoonosis. These include the hepatitis E virus (which causes hepatitis but not gastroenteritis!) which can infect pigs and which can sporadically infect humans *via* undercooked meat, even if it seems to be transmitted preferentially by water (Yugo et al., 2013), and certain coronaviruses infecting bats: the CoV-SARS-2 at the origin of Covid-19 (Yeo et al., 2020) and the SARS-COV (or CoV-SARS-1) causing severe acute respiratory syndrome (SARS) (Riley et al., 2003) which are also enteric viruses found in wastewater (Randazzo et al., 2020; Ahmed et al., 2020; Rimoldi et al., 2020; La Rosa et al., 2020) and which can sometimes cause diarrhoea in infected people (Song et al., 2020).

When they are discharged into the sea with insufficiently treated wastewater, human enteric pathogens can be filtered and retained by bivalve molluscs (oysters, mussels, wedge clams, razor clams, venus clams, tellina, etc.) and transmitted by the latter when they are eaten raw or undercooked (Suffredini et al., 2017; Le Mennec et al., 2017; Loutreul et al., 2014; Pavoni et al., 2013; Fernández and Gómez, 2010). By filtering large volumes of water, bivalve molluscs can retain viruses on/in different tissues (Rincé et al., 2018; Pavoni et al., 2013; Fernández and Gómez, 2010; DePaola et al., 2010; Le Guyader et al., 2009; Boxman et al., 2006; Croci et al., 2005), bacteria (Tamber et al., 2020; Rincé et al., 2018; Senachai et al., 2013; Brands et al., 2005; Gonzalez et al., 1999) and/or unicellular parasites or their oocysts (Rincé et al., 2018; Coupe et al., 2018; Conrad et al., 2005; Giangaspero et al., 2005; Fayer et al., 1997). The concentration of pathogens is such that it can reach levels up to 1000 times higher than in surrounding waters (Love et al., 2010). In connection with the seasonality of certain infections by enteric pathogens in humans, some seafood contaminations may themselves be seasonal (La Bella et al., 2017; Hassard et al., 2017; Le Mennec et al., 2017; Ilic et al., 2017; Rusiñol et al., 2015; Lipp et al., 2001; Curriero et al., 2001; Rose et al., 2000). In all cases, the “deuration” (i.e. the elimination of pathogens retained by molluscs in healthy environments can take from a few days (Love et al., 2010), a few weeks (Polo et al., 2018), to up about 2 months (Suffredini et al., 2017). Retention of pathogens can occur at specific sites and/or in specific tissues; it differs for example between norovirus GI and norovirus GII (Lowmoung et al., 2017; Le Guyader et al., 2012; Maalouf et al., 2010; Fayer et al., 1997). Human enteric viruses are considered to be the main culprits of AGEs transmitted *via* the consumption of bivalve molluscs (Pavoni et al., 2013), or *via* the consumption of crustaceans such as crabs and shrimps which ingest viruses when they eat contaminated oysters or other molluscs (Lekshmi et al., 2018; Suffredini et al., 2008). Their contamination is generally considered to occur almost exclusively during their production (Hall et al., 2012). Sometimes difficult to compare because they correspond to measurements made on variable quantities of whole molluscs or of some of their tissues, the prevalence of norovirus contamination varies in particular with molluscs (Pavoni et al., 2013), their geographical areas of production (Pavoni et al., 2013; Pepe et al., 2012; DePaola et al., 2010; Boxman et al., 2006), and the viruses themselves (DePaola et al., 2010); in Europe, they can be much higher in markets than in production areas, possibly due to supplies from other countries (Schaeffer et al., 2013). But the contaminations can involve other pathogens: Tamber et al. (2020) observed contamination of oysters by *Salmonella enterica* at harvest ($4.3 \log_{10} \text{ cfu.g tissue}^{-1}$) which decreased after 4 days of storage at room temperature ($3.7 \log_{10} \text{ cfu.g tissue}^{-1}$); Ristori

et al. (2007) noted prevalences of 10% in *Salmonella* spp. for samples of 12 oysters each taken from an estuary near Sao Paulo (Brazil), while Martinez-Urtaza et al. (2003) found prevalences of 2.2%, 2.4% and 1.0% in *Salmonella* spp. for oysters, mussels and clams, respectively, taken in mollusc depuration factories in Galicia (Spain); and DePaola et al. (2010) noted a prevalence of 1.5% of *Salmonella* spp. for oysters marketed in nine US states.

Human enteric pathogens can also contaminate fruits and vegetables. Human enteric viruses, in particular human noroviruses, are reported by many authors to be the most frequent pathogen (Callejón et al., 2015; DiCaprio et al., 2015; Díaz et al., 2012; Berger et al., al., 2010). In the US, leafy-vegetables are also the main food vectors of human noroviruses, ahead of fruits/nuts and molluscs (Figure 1) (Hall et al., 2012). The prevalence of norovirus

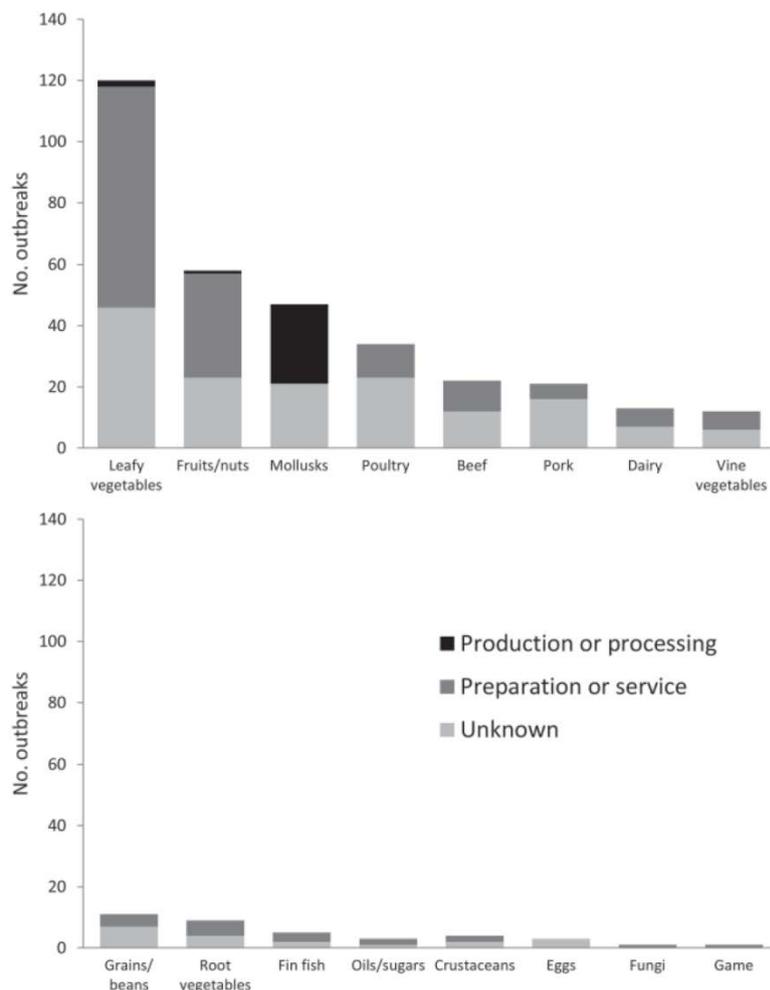


Figure 1: Number of food-borne Norovirus AGEs outbreaks identified in the USA between 2001 and 2008, for different foods according to the contaminating stage (production, preparation and service, unknown) (taken from Hall et al. (2012)).

GI and GII for 25 g samples of fresh salads supplied to the French market by industrial producers in Spain, Italy, Belgium, France and Tunisia were approximately 11%-13% and 0%-1%, respectively. (Loutreul et al., 2014); similarly, the prevalence of norovirus GI and GII of 25 g samples of frozen red fruits supplied to the French market by industrial producers in Serbia, Chile, Bulgaria, Poland, Spain, Morocco, Turkey and France varied between 0% and 50% and between 0% and 3%, respectively. Other data point in the same direction for fresh or frozen red fruits (strawberry, blueberry, raspberry, cranberry, blackberry and blackcurrant) produced in China but with higher prevalence for norovirus GII than for norovirus GI (Gao et al. al., 2019). Furthermore, El-Senousy et al. (2013) found that norovirus GI was present in 20.8% to 34.0% of naturally contaminated fresh vegetables (for 25 g samples). But human enteric pathogens other than viruses such as *Cryptosporidium* spp., non-typhoidal *Salmonella* spp. and shigatoxin-producing *E. coli* (Hoffman et al., 2017) can also contaminate fruits and vegetables. Noroviruses, *Salmonella* spp. and shigatoxin-producing *E. coli* had been identified in 54%, 21% and 10%, respectively, of 972 AGEs clusters associated with the consumption of raw vegetables between 1998 and 2013 in the USA (Bennett et al., 2018). Guchi and Ashenafi (2010) noted prevalences of contaminations of lettuce and other leafy-vegetables at marketing of 10% and 30% for respectively *Salmonella* spp. and *Shigella* spp. for samples of 25 g. In their meta-analysis, De Oliveira Elias et al. (2019) reported a prevalence of 4% for lettuce for both EHEC and *Salmonella* spp. Human enteric pathogens can contaminate foods during production, processing, packaging, storage and during preparation for their consumption (Soto Varela et al., 2016; Sapers and Doyle, 2014; Barak and Schroeder, 2012). We can reasonably think that this result depends in part on food practices, agricultural production methods and everything related to food hygiene (Kundu et al., 2018; Butler et al., 2015; Eraky et al., 2014; Kroupitski et al., 2009; Ukuku and Sapers, 2007; Gagliardi et al., 2003) as with personal hygiene (Dubois-Brissonnet and Guillier, 2020; Donkor et al., 2009), these factors depending themselves on economic constraints (Papadopoulos et al., 2013; Donkor et al., 2009) etc.. Several factors influence their contamination/decontamination along the continuum from farm to fork, such as the quality of irrigation water (Qu et al., 2016; Lynch et al., 2009), adaptation to temperature (Lynch et al., 2009; Huss et al., 2000), as well as the resistance of pathogens to food processing processes, including exposure to antimicrobials (Davidson and Harrison, 2002). During the production of fruits and vegetables, their external contamination varies with the quality of the water and the method of irrigation (Renault et al., 2017; Holvoet et al., 2015; Martínez-Vaz et al., 2014; Gu et al., 2013; Cevallos-Cevallos et al., 2012a; Cevallos-Cevallos

et al., 2012b; Gandhi et al., 2010; FDA, 2008; Chaidez et al., 2005). It can also be affected by wild or domestic animals (Schaefer et al., 2020; Jiang et al., 2015; Jay-Russell, 2013; Avendaño et al., 2010; Allende and Monaghan, 2015) (insects – especially flies involved in the contamination of leaves by *E. coli* O157:H7 (Talley et al., 2009; Iwasa et al., 1999; Janisiewicz et al., 1999) – ...), by the soil which can allow certain human enteric pathogens to retain their infectiousness (Hunt and Johnson, 2017; Tesson and Renault, 2017; Jiang et al., 2015), by dirty equipment and handling with dirty hands (Dubois-Brissonnet and Guillier, 2020; Holvoet et al., 2015; Berger et al., 2010), as well as by spreading manure or compost (Holvoet et al., 2015; Gu et al., 2013). Pathogens can be internalized *via* the stomata of plants (Jacob and Melotto, 2019; Panchal et al., 2016; Berger et al., 2010; Macarisin et al., 2010), but especially via their roots for viruses (Tesson, 2018; Renault et al., 2017) from where they can migrate to raw consumed parts of plants (Tesson, 2018; Renault et al., 2017; Carducci et al., 2015). On the surface as in plant tissue, the binding sites that affect the immobilization of a virus vary with the virus or even with the virus strain for noroviruses (Esseili et al., 2019). However, there are still too few studies on the contamination of leafy vegetables during their production, whether it is external or internal contamination (Renault et al., 2017). Certain items seem very little or not addressed such as the inactivation of viruses on the external plant surfaces, their immobilization in the metaxylem, their exudation with transpiration/guttation, and their inactivation by agents of secondary metabolism (Renault et al., 2017). Note that beyond simple surface or internalized contaminations, Jo et al. (2019) showed that *Shigella* spp. could proliferate in *Arabidopsis thaliana* plants and fresh vegetables. Beyond the harvest, studies highlight the impact of subsequent handling of food (Pérez-Rodríguez et al. 2019; Butler et al., 2015), which would explain most of the contaminations (Hall et al., 2012). Treatments for fresh-cut leafy vegetables are limited (Renault et al., 1994). Chlorine has a positive impact on the inactivation of viruses deposited on vegetables, supplied as chlorinated water (Fuzawa et al., 2019) or as a gas (chlorine dioxide), while Du et al. (2003) show reductions of at least 3 log₁₀ for *E. coli* O157:H7 on the surface of apples. It should be noted that washing promotes cross-contamination (Murray et al., 2017), and that packaging in a modified atmosphere and cold prolong the infectivity of the virus (Baert et al. 2009).

III.3. Fomite transmissions

In Latin, the term fomites referred to the sparks or tinder that made it possible to light or carry fire. Today, in the medical field and concerning AGEs, we speak of fomite or passive vector

of transmission for an object contaminated by a human enteric pathogen and capable of transmitting it to humans directly through contact with the mouth (Lopez et al., 2013; Vasickova et al., 2010; Haas et al., 1999), or indirectly, in particular *via* the hands (Lopez et al., 2013; Haas et al., 1999) or food (Vasickova et al., 2010; Rzeżutka and Cook, 2004). Fomites potentially include kitchen worktops, kitchen utensils and appliances, games, telephones, computer keyboards, door handles, various inert surfaces (walls, windows, etc.), and more generally all objects whose use is shared. At the origin of their contamination, hands poorly washed after contact with faeces (Stobnicka et al., 2018; Barker and Bloomfield, 2000), or even the deposit of aerosols generated by vomiting (La Rosa et al., 2013 ; Caul, 1994) or a deposit of saliva (Fedorenko et al., 2020; Petersen et al., 1976).

Pathogen transmissions by fomites have been observed in day-care centres (Laborde et al., 1993), schools (Bright et al., 2010), offices (Boone and Gerba, 2010), homes (Barker et al., 2001; Speirs et al., 1995), public spaces (Reynolds et al., 2005) and hospitals (Kramer et al., 2006; Huslage et al., 2010; Weber et al., 2010). Several studies have shown that fomites can contribute significantly to enteric infections by viruses (Vasickova et al., 2010), bacteria (Arhin et al., 2020) or unicellular parasites (Innes et al., 2020). The contribution of fomites to the transmission of human enteric pathogens depends on the duration of pathogen survival and maintenance of infectivity. This duration ranges from less than an hour (Yeargin et al., 2016; Sizun and Talbot, 2000; Scott and Bloomfield, 1990) to several weeks (Morita et al., 2011; Wilks et al., 2005; Abad et al., 1994); it depends on microorganisms (Kramer and Assadian, 2014; Kramer et al., 2006; Abad et al., 2001; Abad et al., 1994), on the chemical nature and on the porous nature or not of the fomites (Yeargin et al., 2016; Lopez et al., 2013; Wilks et al., 2005; Abad et al., 2001; Abad et al., 1994), and the environmental context (Abad et al., 2001; Kramer et al., 2006), in particular relative humidity (Alkhamis and Noweir, 2018; Alidjinou et al., 2018; Kramer and Assadian, 2014; Vasickova et al., 2010; Koopmans and Duizer 2004) and air temperature (Alkhamis and Noweir, 2018; Kramer and Assadian, 2014; Vasickova et al., 2010; Abad et al., 2001; Abad et al., 1994), or even organic compounds deposited with pathogens (Garret et al., 2006; Bazaka et al., 2011; Abad et al., 2001; Abad et al., 1994).

Enveloped viruses are inactivated within an hour to a few days on fomites (Boone and Gerba, 2007; Lai et al., 2005; Sizun and Talbot, 2000); this is the case in particular with SARS viruses (SARS-CoV-1) (Poggio et al., 2020; Firquet et al., 2015; Lai et al., 2005) and of Covid-19 (SARS-Cov- 2) (Poggio et al., 2020; Carraturo et al., 2020) which are enteric viruses. Conversely, non-enveloped viruses including most human enteric viruses (norovirus,

rotavirus, enterovirus, adenovirus, astrovirus, etc.) can remain infectious for several weeks to several months on certain fomites (Alidjinou et al., 2018; Stobnicka et al., 2018; Vasickova et al., 2010; Abad et al., 2001), but some materials can inactivate them very quickly (Lai et al., 2005; Abad et al., 1994). Generally speaking, virus inactivation increases with increasing temperatures (Wilks et al. 2005; Abad et al., 2001); it is often faster at intermediate relative air moistures (Verreault et al., 2008; Abad et al., 2001), but this result seems to depend on the virus (Kramer and Assadian, 2014; Verreault et al., 2008). Pathogens other than viruses, including bacteria, can also survive on inert surfaces for long periods (Morita et al., 2011; Kramer et al., 2006; Wilks et al. 2005; Islam et al., 2001a). If stainless steel and certain alloys lead to little or no *E. coli* O157:H7, Cu or alloys with more than 70% Cu (bronzes, brasses, etc.) can cause their disappearance at 20°C in less than 6 h (Wilks et al. 2005). Morita et al. (2011) showed that *Salmonella* spp. survive on threaded surfaces of steel bolts for 336 days at 20-25°C and at relative air moisture of 2-15%. Transfers of bacteria and viruses to the hands are easier from non-porous fomites and at high relative air moisture (Lopez et al., 2013).

Various studies have investigated the effectiveness of fomite disinfection treatments. These treatments can be based on alcohols, detergents and/or oxidants (Barker et al., 2004; Rutala et al., 1998; Sattar et al., 1989), or even sometimes from biocides such as formaldehyde (Rutala et al., 2008; Wolff et al., 2005; Jenkins et al., 1999). The most widely used alcohols are ethanol and isopropyl alcohol, generally at 70% (Wolff et al., 2005; Vieira et al., 2005; Duizer et al., 2004; Saknimit et al., 1998; Wood et Payne, 1988; Brown, 1981). For example, *E. coli* and *Salmonella* spp. were killed in 10 s by all solutions containing between 40% and 100% ethanol (Rutala et al., 2008). Chlorine, applied in the form of sodium hypochlorite for a few minutes, allowed a very significant reduction in *Salmonella* spp. (Rutala et al., 2008; Rutala et al., 1998) and norovirus (Barker et al., 2004). But in some cases, it is the succession of treatment with a detergent and then with an oxidant (hypochlorite) that is necessary to completely disinfect contaminated surfaces (Barker et al., 2004). Other oxidants are also effective such as peracetic acid (Rutala et al., 2008). Some pathogens exhibit forms of resistance, such as oocysts of *Cryptosporidium* spp.; they have the ability to withstand temperatures <60°C (Hotchkiss et al., 2015) and commonly used disinfectants (King and Monis, 2007) and thus have an increased possibility of survival in the environment (Innes et al., 2020). Ammonia used at acidic pH can inactivate these oocysts (Vermeulen et al., 2017; Jenkins et al., 1999).

III.4. Air transmission

Airborne outbreaks of viral AGEs have been reported in indoor environments (Nazaroff, 2011), for example in restaurants where the rate of infection per table was inversely proportional to distance from a person who vomited (Marks et al., 2000), in schools (Marks et al., 2003) and in hospitals (Alsvéd et al., 2020; Wan et al., 2012). The air of indoor environments can be contaminated with pathogens other than viruses, for example by *Salmonella* spp., *Shigella* spp., *E. coli* and/or *Bacillus subtilis* for households in Zaria (Nigeria) (Abe et al., 2012), by *Salmonella* spp., *Shigella* spp., *E. coli* and fungi for contaminated industrial poultry houses (Yasmeen et al., 2020), and *Shigella boydii* for poultry houses (Long and Plewa, 2011).

In the vicinity of the latest buildings, outdoor air can also be contaminated, but at lower levels (Yasmeen et al., 2020; Long and Plewa, 2011). In addition, during windy periods, human enteric viruses (Masclaux et al., 2014) as well as coliforms and *Enterococcus* spp., *E. coli* and *Staphylococcus* spp. (Brandi et al., 2000) have been detected in outside air in and near wastewater treatment plants; but pathogens such as *Salmonella* spp., *Shigella* spp., *Pseudomonas aeruginosa* and *Aeromonas* spp. were not detected in the last cited study (Brandi et al., 2000). Beyond these 2 studies, we summarize in **Table 5** the main results of a few monitoring campaigns in wastewater treatment plants and landfills. Concerning crops, studies have observed the aerosolization of *E. coli* in the vicinity of sprinklers in cultivated fields irrigated by wastewater (Teltsch and Katzenelson, 1978), the presence of human enteric viruses in the air in the vicinity of areas irrigated by wastewater (Courault et al., 2017), and the possibility for viruses deposited with contaminated water on the soil to be aerosolized later in windy conditions (Girardin et al., 2016).

However, the existence of viral AGE outbreaks initiated by transmission through the air in an outdoor environment remains to be demonstrated, as does the impact on human health of the outdoor aerosolization of enteric viruses from contaminated surfaces or resulting directly from irrigation with contaminated water which has been little studied (Courault et al., 2017; Girardin et al., 2016). To our knowledge, only two studies have reported the infectivity of human enteric viruses in outdoor air, namely adenoviruses in the air of a recycling area of a landfill that may have infected the A549 cell line (ECACC) (Carducci et al., 2013; Carducci et al., 2009). However, quantitative microbial risk assessments (QMRA) have already been proposed for airborne transmission in outdoor environments (Courault et al., 2017).

Sampling location	Human pathogens and indicators of faecal contamination						Reference
	NV ²	RV-A ³	AdV ⁴	<i>E. coli</i>	C. tot. ⁵	SI/Sh ⁶	
	----- CG.m ⁻³ -----			---- UFC.m ⁻³ ----			
WWTP ¹ (activated sludge basin)	3.1 10 ⁺³	2.7 10 ⁺¹	-	-	-	-	Pasalari et al. (2019)
WWTP ¹ (activated sludge basin)	< 10 ⁺³	< 10 ⁺⁴	-	-	-	-	Courault et al. (2017)
WWTP ¹ (activated sludge basin)	<6.5 10 ⁺²	-	>22.7 10 ⁺⁷	-	-	-	Masclaux et al (2014)
WWTP ¹ (activated sludge basin)	-	-	2.9 10 ⁺⁰	-	-	-	Carducci et al. (2016)
Landfill (outdoor areas)	-	-	5.1 10 ⁺⁷	-	-	-	Carducci et al. (2013)
Landfill (waste compaction site)	-	-	2.7 10 ⁺⁰	-	-	-	Carducci et al. (2016)
WWTP ¹ (pre-treatment: screening)	-	-	-	95	63	205	Azevedo et al. (2014)
WWTP ¹ (activated sludge basin)	-	-	-	15	15	15	Azevedo et al. (2014)
River downstream of the discharge of treated wastewater	-	-	-	95	25	40	Azevedo et al. (2014)

¹WWTP: Wastewater treatment plant; ²NV: Norovirus; ³RV-A: Rotavirus type A; ⁴AdV: Adenovirus ; ⁵C. tot.: total coliforms; ⁶Salm/Shig: *Salmonella* spp. + *Shigella* spp.

Table 5: Concentrations of human enteric pathogens and indicators of faecal contamination in the air above wastewater treatment plants, landfills and rivers.

III.5. Indicators of faecal contamination; interests and limits

Direct monitoring of the concentrations of human enteric pathogens is not necessarily easy for various reasons: their multitude, the often high cost of enumerating them, sometimes the time required for this assessment, and their dangerousness.

Therefore, various indicators of faecal contamination are often used in research dealing with microbiological risks for humans (Rodrigues et al., 2011; Nagvenkar and Ramaiah, 2009) and/or for regulatory monitoring (Banach and van der Fels-Klerx, 2020), including the microbiological quality of drinking water (US-EPA, 2017; WHO, 2011; Min.Salud - Min.Ambiente, 2007; European Commission, 1998), recreational waters (Leonard et al., 2015), and wastewater leaving the wastewater treatment plant before discharge into the river (Ottoson et al., 2006; Payment et al., 2001) or before reuse in agricultural irrigation (MASS (2014) for example). Indicators of faecal contamination include *E. coli*, faecal coliforms, total coliforms, total or faecal *Enterococci* (Uyttendaele et al., 2015; WHO, 2006; Paz-y-Miño et al., 2003), and sometimes other indicators, such as F-specific RNA phages (i.e. viruses of

E. coli and *Salmonella* spp. in particular) (MASS, 2014). When involved in regulations relating to the reuse of wastewater in agricultural irrigation, the standards depend on the destination of the irrigated products (Banach and van der Fels-Klerx, 2020; Uyttendaele et al., 2015), but these standards differ strongly between countries (Renault et al., 2013), and sometimes even between States or Regions of the same country (Keraita et al., 2008). While WHO (2006) recommended faecal coliform concentrations lower than 10^{+3} per 100 mL to irrigate vegetables consumed raw, some countries of the European Union have adopted very different thresholds for use in “unrestricted irrigation”: $<250 E. coli.(100 mL)^{-1}$ in France according to a 2010 decree, $<100 E. coli.(100 mL)^{-1}$ in Spain according to a 2007 Royal decree, $<10 E. coli.(100 mL)^{-1}$ in 80% of samples according to a 2003 decree in Italy, and <5 faecal coliforms.(100 mL)⁻¹ in 80% of samples in Cyprus for small agglomerations according to a 2005 decree (Renault et al., 2013). In Colombia, the irrigation of fruits and vegetables that are consumed raw can use waters having up to 10^{+3} faecal coliforms.(100 mL)⁻¹ and up to $5 \cdot 10^{+3}$ total coliforms.(100 mL)⁻¹ (República de Colombia, 1984).

E. coli is probably the most commonly chosen indicator of faecal contamination (Tesson et al, 2018). It is very representative of faecal coliforms present in the intestinal microbiota of humans and is more specific for faecal contamination of water than other species of faecal coliforms (Khan and Gupta, 2020) and coliforms other than faecal coliforms (Leclerc et al., 2001); it is currently used as an indicator for faecal contamination of drinking water, due to its specificity and the existence of simple, inexpensive, rapid, sensitive and precise detection techniques (Khan and Gupta, 2020). It can survive in water for several weeks (Khan and Gupta, 2020) but, unlike total coliforms other than faecal coliforms, it is often more likely to survive than to live and grow in the environment (Leclerc et al., 2001). Among the other commonly selected faecal indicators, enterococci are of interest in being relatively resistant to certain treatments (temperatures, antibiotics) (Sood et al., 2008) and in the environment (García-Solache and Rice, 2019). Finally, the F-specific RNA phages which infect *E. coli* and *Salmonella* spp. in particular (and which are therefore systematically present in domestic wastewater) are used as a model of human enteric viruses for standard and regulation (MASS, 2014)).

Researches have been dedicated to the indicators of faecal contamination, including their fate in the environment (Heliodoro and Bustos-López, 2018; Tallon et al., 2005; Leclerc et al., 2001). But they are often monitored alongside human enteric pathogens (Murphy, 2017; Nagvenkar and Ramaiah, 2009; Rose et al., 1996). The presence of indicators of faecal contamination indicates the potential presence of human enteric pathogens: Rodrigues et al.

(2011) observed in polluted estuarine waters correlations between on the one hand the concentrations of *E. coli*, faecal coliforms and total coliforms, and on the other hand the concentrations of *Shigella* spp. and *Salmonella* spp.; similarly, Nagvenkar and Ramaiah (2009) noted relationships between concentrations of *E. coli*, total coliforms, faecal coliforms, *Vibrio cholerae* and *Shigella* spp. in estuarine waters.

But the link between the concentrations of faecal indicators and the concentrations of human enteric pathogens is not always obvious for various reasons and several authors have considered these indicators to be of little relevance (Tesson et al., 2018; Payment and Locas, 2011; Paz-y-Miño et al., 2003). In particular, most of the studies that have followed human enteric viruses and indicators of faecal contamination at the same time have concluded that there is no correlation between the concentrations of both (Prevost et al., 2015; Pepe et al., 2012; Paz-y-Miño et al., 2003; Griffin et al., 2003; Jiang et al., 2001; Borrego et al., 1987). The frequent lack of correlation between concentrations of human enteric pathogens and concentrations of indicators of faecal contamination is due to several reasons: (i) indicators of faecal contamination indicate faecal pollution which may not be concomitant with the presence of enteric pathogens when the pollution comes from a population without any infected person, (ii) the indicators of faecal contamination are not specific to humans but at least to the intestinal microbiota of all mammals, and (iii) the non-conservative behaviour of faecal contamination indicators and human enteric pathogens in the environment, including wastewater treatment plants, can lead to "de-correlating" the concentrations of each other, in particular as a function of contexts that may be more favourable to one than to the other (Cook and Bolster, 2007). Thus, by way of example, the removals in *E. coli* and in *Salmonella* spp. in groundwater differed from each other (Sidhu et al., 2015), as did the removals of *E. coli* and *Campylobacter jejuni* in other groundwater (Cook and Bolster, 2007). In the extreme, we may have water without indicator (s) of faecal contamination but with enteric pathogens, for example viruses (Rose et al., 2006; Fattal et al., 1983).

IV. Measurement of pathogen concentrations in water, food and air

IV.1. Trapping, concentration and purification of viruses in water

The detection and enumeration of human enteric viruses present in water compartments (river, aquifer, sea, etc.) are generally carried out after a succession of steps aimed at concentrating

the viruses and purifying their suspension liquid to be over the detection thresholds of the enumeration methods and minimize the artefacts specific to these methods (inhibitions in molecular methods (qPCR and RT-qPCR), inactivation for the enumeration of cell culture lysis plaques, etc.). These processes can relate to quantities of water ranging from approximately 1 L (or less) for bottled water checks (Schultz et al., 2011) or for wastewater (Tesson et al., 2019) to several hundred Liters for aquifer or spring water (Lodder et al., 2010), passing through intermediate quantities for river water (100 L (Tesson et al., 2019), 20 L (Prevost et al., 2015), 10 L (Hamza et al., 2009) , 2 L (Teixeira et al., 2017), 0.5 L (Haramoto et al., 2005) for examples).

Therefore, these processes often start with a step of immobilization of the viruses – which are generally negatively charged at current water pH – on a positively charged filter such as NanoCeram® filters (Tesson et al., 2019; Prevost et al., 2015), on positively charged soda lime glass wool or powder contained in a cartridge (Lambertini et al., 2008; Vilaginès et al., 1993; Hugues et al., 1992), or on a negatively charged filter such as the so-called HA filters in mixed cellulose ester (Millipore®) after having either doped them with di- or trivalent cations (Mg^{2+} , Al^{3+} in particular) (Haramoto et al., 2005; Haramoto et al., 2004) or modified the virus charge by adding di- or trivalent cations to contaminated water, the pH of which is sometimes simultaneously adjusted (Hamza et al. 2014; Lodder et al., 2010). The elution of viruses from filters depends on the nature of the filters. It is carried out in several steps for HA filters with first the substitution of di- or trivalent cations allowing virus-filter bonds by protons (Hamza et al. 2014; Lodder et al., 2010; Haramoto et al., 2004). The nature of the compounds used to elute viruses is partly determined by the subsequent detection: the use of beef extract is recommended for maintaining the infectivity of the viruses, while this product seems to inhibit qPCR molecular methods (Lodder et al., 2010; Bessetti, 2007). This filtration-elution step is very often followed by a flocculation-centrifugation step. Flocculation is very often caused by polyethylene glycol (Lodder et al., 2010; Lambertini et al., 2008), in particular if the subsequent detection is carried out by molecular method. Beef extract is sometimes preferred, which seems to better preserve the infectivity of viruses (Katayama et al., 2002). After centrifugation, the dispersion of the pellet in a phosphate buffered saline (PBS) or other medium may be the last step before the start of the enumeration steps, but the purification can also be continued by other operations, in particular ultracentrifugation on sucrose cushion (Tesson et al., 2019). Other methods have emerged in recent years, including the use of tangential ultrafiltration which is sometimes applied to raw water samples for faster virus purifications and less handling, as is currently the case for SARS-CoV-2 (Lu et al., 2020).

IV.2. Trapping, concentration and purification of viruses in the air

Different methods have been proposed to assess the concentrations of viruses in the air or, more generally, of human pathogens (Girardin, 2015; Verreault et al., 2008). They are based on the use of different types of biocollectors to trap pathogens. These biocollectors include filters (Clark Burton et al, 2007), possibly used passively (i.e. without imposed air flow, the filters used being just placed in Petri dishes exposed to air) (Courault et al., 2017), liquid impactors (or impingers) (Han and Mainelis, 2012), cyclonic-type biocollectors (Le Goff et al., 2011), and Andersen-type cascade impactors allowing particle size separation of bioaerosols (Zhao et al., 2014; King and McFarland, 2012). These allow *a posteriori* characterization of bioaerosols (aggregation of viruses, size and shape of inert particles to which they can adhere, etc.) but, until now, few studies report a precise quantification of the particle size distribution of aerosols above agricultural surfaces, associated with a characterization of bioaerosols made by microbiological analyses. The remaining operations depend on the virus trapping mode. It may be subject to the same limits as those for samples taken in water for trapping in solution and/or as those for samples taken from the surface of plants (see sub-section IV.3).

IV.3. Elution, concentration and purification of viruses from fruit and vegetable external surface

The protocols used to enumerate the viruses immobilized on the external surfaces of fruits or vegetables all start with their elution which is followed by various concentration and purification operations, the latter possibly being close to or identical to those already mentioned in the sub-section IV.1.

Several procedures for eluting viruses immobilized on the surface of vegetables or fruits have been proposed by Sadovski et al. (1978a, 1978b), Ward et al. (1982), Gouvea et al. (1994) and Dubois et al. (2002). The ISO/TS15216-1 (ISO, 2012) standard has since proposed an initial elution procedure very close to that proposed by Dubois et al. (2002); it has been used in numerous studies, and has given rise to an inter-laboratory evaluation (Lowther et al., 2019). In all of these studies, viruses were eluted from fragments of contaminated fruits or vegetables by shaking these fragments in an eluting solution at alkaline pH (Table 6). Note that for food surfaces (kitchen worktops, etc.), the ISO/TS15216-1 standard proposes a fairly different method based on passing a swab soaked in PBS from which the viruses will then be

Vegetable	Quantity (g fresh matter)	Solution/Elution buffer	Stirring time	Number of consecutive elutions	Reference
Cucumber	500-1000 g	500 mL of 0.85% NaCl in 0.02 M phosphate buffer, pH 8.0)	7 min	some (imprecise)	Sadovski et al. (1978a)
Cucumber	800 or 1400 g	1000 mL of 0.85% NaCl in 0.02 M phosphate buffer, pH 8.0)	7 min	2	Sadovski et al. (1978b)
Cabbage	4.5-9.8 kg	2 L PBS, pH 9 used in 2 buckets successively (4 à 24 buckets per sample)	7-10 min	1	Ward et al. (1982)
Melon, Lettuce	10 g	10 mL 6 M guanidinium thiocyanate (GITC)	1 min	2	Gouvea et al. (1994)
Raspberries, Strawberries, Blueberries, Blackberries, Blackcurrant, Cherry, Lettuce, Radish, Tomatoes	30-100 g	300 mL of an elution buffer (100 mM Tris-HCl, 50 mM glycine, et 3% d'extrait de bœuf, pH 9.5) with 50 mM de MgCl ₂ for infectivity tests	20 min	1	Dubois et al. (2002)
Red fruits, salades	25 g	40 mL TGBE buffer (100 mM Tris, 50 mM glycine, and 1% beef extract, pH 9.5)	20 min	1	ISO/TS15216-1
Food surface	≤100 cm ²	Cotton swab moistened with PBS	-	1	ISO/TS15216-1

Table 6 : Main characteristics of the initial phase of elution of viruses retained on the surface of vegetables or red fruits.

counted (ISO, 2012). The amounts of plant material could vary from 10 g to 9.8 kg; while small quantities can affect the variability of the results (and affect the prevalence for samples taken *in situ*), several of the studies mentioned here started with artificial inoculation of the samples, limiting for these studies the scope of this note. Obviously, working on small quantities (25 g of salad as recommended in the ISO/TS15216-1 standard) can lead to insignificant results if they are not repeated on a large number of samples, in particular if the contamination results from handling after harvest.

IV.4. Variants for bacteria and unicellular parasites

From a methodological point of view, viruses are distinguished from other human enteric pathogens (bacteria, unicellular parasites, helminths) or from certain forms of “resistance” of the latter (for example, spores for *Clostridium perfringens* and *Bacillus cereus*, oocysts for *Cryptosporidium* spp. and helminth eggs) by their size (20-80 nm for viruses, against 0.3-6 µm for *Salmonella* spp. and *Shigella* spp., and 4-6 µm for *Cryptosporidium* spp.) and the inability to cultivate viruses without allowing them to infect target cells. These differences in size and cultivability result in procedures that are often more complex for viruses than for

other human pathogens.

When they are in water or in the air, they can be filtered very generally on size criteria (Gray, 2014; Sundaram et al., 2001) and allow an enumeration on filter based on cultivation of bacteria (Weir et al., 2001; Barer and Harwood, 1999) or unicellular parasites (Slifko et al., 1997; Gottschal et al., 1992), on the use of specific detection possibly linked to the molecular biology (for example hybridization of 18S rRNA in *Cryptosporidium parvum* (Rochelle et al., 2001)) or linked to plasmid DNA (Olson et al., 1999), or an enumeration out of filter after elution of either pathogens from filters on which they were trapped (Mortimer et al., 2016; Kerrouche et al., 2015) or of some of their constituents – in particular their DNA (Francy et al., 2013; Kaushik and Balasubramanian, 2012) – after the disintegration of the pathogens by treatment with chloroform for example (Vanhuynh et al., 1993).

When initially immobilized on food, pathogens can be concentrated and purified after an elution or grinding phase by various techniques, including density gradient separation (Lindqvist, 1997). Dilutions may be necessary in order to have a correct enumeration (Wiegand et al., 2008).

IV.5. Detection and enumeration

There are various methods for detecting human enteric pathogens. They depend on the pathogens to be detected, the concentration ranges at which they are found and the information expected (total quantities or infectious quantities, virulence, diversity, etc.).

When we are interested in total concentrations and more specifically for viruses, molecular methods (qPCR and RT-qPCR), possibly multiplexed and then allowing the simultaneous detection of several pathogens (Hamza et al., 2014; Baums et al., 2014; Baums et al. al., 2007), are probably the most sensitive (Toze, 1999): they are adapted to very low concentrations, but they have a lower relative precision (Ramanan et al., 2017, Sue et al., 2014; Lindsay et al., al., 2013 Karim et al., 2009; Haramoto et al., 2004) and potentially present biases due to interferences between primers (Hamza et al., 2014), fluorescent chemicals (Huang et al., 2011) and PCR inhibitors (Bessetti, 2007). Their specificity depends on the RNA or DNA sequences used for their recognition (Lindsay et al., 2013; Kirby and Iturriza-Gómara, 2012; Gonzales et al., 2006; Wolfaardt et al., 1995). One of their major limitations is that they do not provide access to the infectiousness of pathogens. However, we should point out some recent developments that make it possible to approach it for viruses by indirectly evaluating the integrity of the capsid by pre-treating the particles with monoazides

(EMA, PMA, PMA_{xx}) (Prevost et al., 2016) with improvements recently made by Lee et al. (2018) for situations where the genetic material of the virus can also be damaged (following UV or chlorine treatment). Other molecular methods can be used, such as the Dot-Blot hybridization method (Cheley and Anderson, 1984). Molecular methods of the qPCR type can of course be used for pathogens other than viruses (Elizaquível et al., 2012; Rochelle et al., 1997).

The infectiousness of pathogens can be assessed when they can be cultivated directly – especially for bacteria and unicellular parasites (Visvesvara and Garcia, 2002; Jakubowski et al., 1996; Hill et al., 1986; Monod, 1949) –, or indirectly *via* cells that they can infect – in particular for viruses (Carducci et al., 2013; Guerrero et al., 2010; Peláez et al., 2010; Carducci et al., 2009) –. It should be noted that until now there has been no method of culturing human noroviruses, although some attempts have been proposed (Oka et al., 2018; Jones et al., 2015; Karst et al., 2015; Karst et al. al., 2014; Taube et al., 2013; Straub et al., 2011; Lay et al., 2010; Duizer et al., 2004; Wobus et al., 2004). While some methods have only been used to indicate the presence or absence of pathogens (Peng et al., 2002), most allow counts based either on the enumeration of colonies developing on a culture medium (cfu for colony forming unit) (Maheux et al., 2014), or on the estimation of the so-called most probable number when one counts positive “wells” and negative “wells” at different dilutions (MPN for Most Probable Number in English) (Feng et al., 2002), or on lysis plaques when viruses infect a mat of previously cultured cells (Oaks et al., 1985).

Other detection methods exist, some of which being quantitative. They may require working on high concentrations of pathogens. Among these, we can distinguish measurements by transmission electron microscopy (Curry et al., 2006), immunological-type methods such as the ELISA test which can be moderately or very specific (Kirby and Iturriza-Gómara, 2012) and too laborious for routine laboratory use (García, 2006). More specifically for the oocysts of certain unicellular parasites, mention may be made of immunofluorescence tests after microfiltration and separation of oocysts from other particles present in the medium (Jakubowski et al., 1996).

V. Conclusion

In this bibliographical review, we have summarized some generalities on AGEs, as well as on the main experimental methodologies allowing to enumerate the pathogens which cause

AGEs from different matrices (air, water, food), but we especially put forward the state of knowledge relating to indirect transmissions of human enteric pathogens *via* food, water, air, and inert surfaces which may be contaminated, in particular following handling with soiled hands.

This review allowed us to describe the main epidemiological characteristics of AGEs. They can be caused by a wide variety of human enteric pathogens found in a wide variety of latitudes, the clinical symptoms and severity of AGEs being dependent on the pathogens involved. Their transmission can be direct or indirect *via* the environment, in particular *via* food, water, inert surfaces, and air at least indoors (homes, offices, schools, hospitals, etc.). If AGEs have very diverse aetiologies, it is now accepted that viral AGEs predominate, and that noroviruses are a major cause. The foods potentially involved in the transmission of AGEs are first those eaten raw with in particular the leafy vegetables which would be more often contaminated by post-harvest handling than during their production, as well as the seafood contaminated mainly during their production. There is a dependence on the regions of the world which probably results from various factors including the climate, individual and collective practices in terms of food, hygiene and health, and which are in part associated with the history and culture of regions/countries, and economic and regulatory contexts. The contamination of wastewater discharged into the environment strongly depends on its treatment (absence/presence, level), and the use of fresh water depends on its use (drinking, recreational use) and its prior treatment. Airborne transmission in an outdoor environment remains uncertain and would depend largely on the survival of aerosolized pathogens.

This review also allowed us to show that many questions of a methodological, scientific or applied nature remain unanswered at present. In particular concerning the methods, we do not know how to measure the infectiousness of human noroviruses despite culture tests and molecular biology proposals to assess their integrity. At the scientific level, we still lack a good vision of the respective contributions of food, water and outdoor air to the indirect transmission of enteric pathogens to humans, this good vision requiring sector-type approaches with regard to food (i.e. from production to consumption) and, more specifically for Colombia, taking into account the risk of transmission through the air of pathogens deposited in landfill with excrements on toilet papers and on babies and adults' diapers. Finally, at a more applied level, the link with the Human and Social Sciences (SHS) still seems little made and probably focused on the punctual analysis of epidemics or contaminations, in particular of water. This link could be made at different levels: practices in terms of food, hygiene and health, communications and persuasion, health economics....

Also, this PhD pursued 3 objectives: (i) the development of a culture method for human norovirus (cf. **Chapter III**), (ii) the characterization of water contamination, leafy vegetables and the air by various enteric human pathogens (viruses, bacteria and unicellular parasite) in the suburbs of Bogotá (Colombia) (cf. **Chapter IV**), and (iii) the possible detection of relationships between living standards, individual practices and cases of AGEs (cf. **Chapter V**).

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Chapter III:

Contamination of water, leafy-vegetables and air by human enteric pathogens (GI and GII noroviruses, rotavirus type A, *Salmonella* spp., *Shigella* spp., *Cryptosporidium* spp.) in the suburb of *Bogotá* (Colombia)

This chapter should give rise, in reduced form, to the submission of the article

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Abstract

Acute gastroenteritis can affect a few tens of percent of the global population each year and is responsible for significant morbidity, mortality and healthcare costs. The relative importance of direct person-to-person transmission depends on its aetiology and the country. In Colombia, indirect food and waterborne transmissions may be favoured by the frequent lack of adequate treatment of wastewater, the irrigation of vegetables and fruits sometimes by contaminated surface freshwater, and the landfill disposal of soiled toilet papers and diapers which may favour pathogen aerosolization. The objective of this work was to enrich the characterization of water, air, and vegetable contaminations by faecal indicator and human enteric pathogens in *Bogotá* and its surroundings. During low and high rainfall months, the concentrations of *E. coli*, total coliforms and human enteric pathogens (norovirus, rotavirus, *Salmonella* spp., *Shigella* spp. and *Cryptosporidium* spp.) were monitored in water bodies (river, storm channel, lixivate from landfill, irrigation water, tap water), leafy vegetables (chards) from production to purchase by end consumer, and air, using cell cultivation and molecular methods. Climatic data (wind, rainfall, and temperature), additional *E. coli* and total coliform concentrations, and surfaces of wastes not covered by clay or soil in the landfill were kindly given by stakeholders. Concentrations of coliforms and human enteric pathogens were analysed with regard to water bodies, climate, urbanisation and irrigation practice; a simple model was proposed to describe chard contamination. Faecal contamination was observed for surface freshwaters in which are discharged raw or insufficiently treated wastewaters. The discharge of wastewater into the *Bogotá* river, the *Balsillas* river and the *Tres Esquinas* marsh leads to the contamination of the irrigation network of *La Ramada* area northwest of *Bogotá* by *E. coli* and potentially pathogens responsible for acute gastroenteritis. *E. coli*, other coliforms and *Shigella* spp. were detected in about 25%, 54% and 63% of 4 m³ air samples; their concentration in the atmosphere apparently depended on the direction of the wind and the rains of the previous days, without making it possible to identify the landfill as a privileged place of microorganism aerosolization. During production, the contamination of chards resulted from their irrigation and/or washing by fresh surface water contaminated by raw or insufficiently treated wastewater. Subsequent washing of chard with tap water at the main wholesale market in *Bogotá* removed 1 to 3.5 log₁₀ of faecal indicator, but chards can be contaminated again on merchant's stall as a result of handling by various customers. A model enables to assess initial contamination of chards during production, at harvest and after

washing in the transporting truck. It is very likely that the risks will decrease with the construction in progress of new wastewater treatment plants and sewer systems.

I. Introduction

Acute gastroenteritis (AGE) affects a significant proportion of the global population each year: about 33% of the French population (Van Cauteren et al., 2012), 55% of the USA population (Lively et al., 2018), and probably more in Colombia, Brazil, Peru and Mexico in view of the data of Troeger et al. (2017) concerning only acute diarrhea, although data for France and the US are surprisingly low in this last work. They are responsible for significant morbidity, mortality and healthcare costs (Troeger et al., 2018; Hoffman et al., 2015). In Colombia, Brazil, Peru and Mexico, the annual mortality of children <5 resulting from acute diarrhea would be around 1 in 1000 (Troeger et al., 2017; Ministerio de Salud y Protección Social, 2019), that is to say around 10 times more than in France or in the US (Troeger et al., 2017). Health costs vary with the aetiology of AGE (Hoffman et al., 2015), as well as the age, health status and access to care of infected persons (Atchison et al., 2016; Ruiz-Rodríguez et al., 2012). Human AGE pathogens include viruses, bacteria and unicellular parasites (Dennehy, 2019). They are found in various climatic contexts (WHO, 2015; Petersen et al., 2011). Viral AGEs generally dominate (Dennehy, 2019) and are often caused by noroviruses in Latin America (Da Silva Poló et al., 2016) and Colombia (Fernandez et al., 2015), as elsewhere, especially since the availability of rotavirus vaccines (López-Medina et al., 2018; Peláez-Carvajal et al., 2014; De la Hoz et al., 2010).

Although it is recognized that direct faecal-oral transmissions often predominate in primary transmissions of AGE, sporadic cases and outbreaks have shown the importance of foodborne transmission (Lively et al., 2018; Soto Varela et al., 2016; WHO, 2015), waterborne transmission (de Deus et al., 2019; WHO, 2015), and airborne transmission at least indoors (Nazaroff, 2011). Indirect transmissions vary with the pathogens (WHO, 2015) according to their survival or maintenance of their infectivity in the environment, the existence of animal reservoirs (O'Brien, 2006) and the ingested dose (WHO, 2015). Indirect transmissions therefore vary with the region. For the 'AMR B' geographical area, which includes most Latin American countries, direct transmissions would account for about 72%, 15%, 51%, and 35% of the transmissions of *norovirus*, non-typhoid *Salmonella enterica*, *Shigella* spp. and *Cryptosporidium* spp., respectively (WHO, 2015). In the same geographical

area, foodborne transmissions would represent approximately 14%, 49%, 14% and 11%, while waterborne transmissions would represent approximately 6%, 9%, 27% and 26% of the transmissions of the same pathogens, respectively (WHO, 2015). To our knowledge and to date in Colombia, there is no estimate of the relative contributions of raw eaten foods to foodborne transmission of AGE; and of the prevalence of human enteric pathogens in food. In the USA, leafy-vegetables explain most of foodborne norovirus AGE outbreaks (Hall et al., 2012), with more frequent contaminations of them during preparation/service than during production/processing, although the source of contamination remains unidentified in a third of cases (Hall et al., 2012). Handling with soiled hands explains most of post-harvest contaminations (Pérez-Rodríguez et al., 2019).

The Colombian context is specific to several points of view. First, around 2016, less than 50% of the Colombian municipalities had wastewater treatment plants (WWTPs) (Gobierno de Colombia, 2018), of which about 17% perform only primary treatment corresponding to about 43% of the Colombian wastewaters (Rodríguez Miranda et al., 2016). The lack of treatment probably contributed to the observed contaminations of tap waters by viruses and unicellular parasites (Peláez et al., 2016; Lenis et al., 2012; Moreno et al., 2009; Gutiérrez et al., 2007; Alarcón et al., 2005) but the situation should evolve with recent Colombian regulations regarding WW discharges and the prevention of drinking water contamination (Costa Posada et al., 2015). Second, the intake of vegetables and fruits is largely restricted to a handful of relatively cheap products (Guarín, 2013) that may be irrigated by surface freshwaters heavily contaminated with faecal indicators (Heliodoro and Bustos-López, 2018) and human enteric pathogens (FAO, 2017). However, the intake of vegetables and fruits in Colombia ($35 \text{ kg.p}^{-1}.\text{y}^{-1}$) is well below that in neighbouring countries like Venezuela ($84 \text{ kg.p}^{-1}.\text{y}^{-1}$) and Mexico ($73 \text{ kg.p}^{-1}.\text{y}^{-1}$) (Guarín, 2013). Third, landfill disposal of stained toilet papers and diapers for babies and adults carries potential risks of pathogen aerosolization (Girardin et al., 2016).

Although some quantitative microbial risk assessments (QMRA) have been carried out in Colombia to assess AGE risks resulting from water drinking (Delgado et al., 2019) and the consumption of raw vegetables (Henao-Herreño et al., 2017), they are supported by too little data dealing with microbial contaminations of water (Heliodoro and Bustos-López, 2018), food (Soto Varela et al., 2016) and air (Tolozza-Moreno and Lizarazo-Forero, 2011; Giraldo-Castrillón et al., 2009). Such QMRAs also requires knowledge on individual food, hygiene and/or health practices that are poorly described in Colombia; they must take into account foreseeable increase in water quality with future developments of WW treatment (Costa

Posada et al., 2015), more distant supplies of agricultural food products (although not concerning leafy vegetables) with higher temperatures and precipitations in the *Bogotá* region (Eitzinger et al., 2014), and maybe an increase in vegetable consumption.

The objective of this work was therefore to enrich the characterization of the contaminations by faecal indicators and human enteric pathogens of water, air, and vegetables from production to purchase by end consumers in *Bogotá* and in its surroundings. Chard was chosen as a model of leafy vegetables eaten raw. *E. coli* and other coliforms were used as indicators of faecal and environmental contaminations, respectively (Tallon et al., 2005; Leclerc et al., 2001). Pathogens monitored include viruses (noroviruses, rotaviruses), bacteria (*Salmonella* spp., *Shigella* spp.), and unicellular parasites (cryptosporidium spp.).

II. Materials and Methods

II.1. Sampling zone

The study focused on some neighbourhoods of *Bogotá* city, the capital of Colombia, and its surroundings. The city is at about 2640 m above sea level. It has a subtropical highland climate (Cfb in the Köppen-Geiger climate classification). For the period 1971-2000, the average monthly temperature varied between 14.1°C and 15.0°C and the average annual rainfall was 1013 mm, with precipitation each month but more than 100 mm per month during April-May and October-November (data from the *Instituto de Hidrología, Meteorología y Estudios Ambientales*). Over the sampling period, monthly average temperatures were about 1°C higher and annual precipitation was 120 to 340 mm higher (Figure 1) due to year-to-year variability and possibly climate change (Moreno and Perdomo, 2018; Eitzinger et al., 2014). *Bogotá* currently stretches about 35 km from north to south and 20 km from west to east (Figure 2). It and the 17 surrounding municipalities have about 8.6 million inhabitants (Rojas et al., 2015). The city has 20 districts, including *Usme* district (about 343 000 inhabitants in 2018 (Alcaldía Mayor de Bogotá, 2018)) that includes *Granada Sur*, *Quintas del Plan Social* and *La Aurora* neighbourhoods (having population densities of about 161, 161 and 227 inhabitants.ha⁻¹, respectively (SDP, 2018)). The city has been established gradually with major steps linked to the conquest of flooded areas, the huge increase in population density and size with informal low-cost urbanization and housing and annexation of surrounding municipalities (including part of *Usme*), and the recent policy for water management due to

Public Health consideration and lifestyle issue (Peña-Guzmán et al., 2017; Rojas et al., 2015; Rojas, 2013).

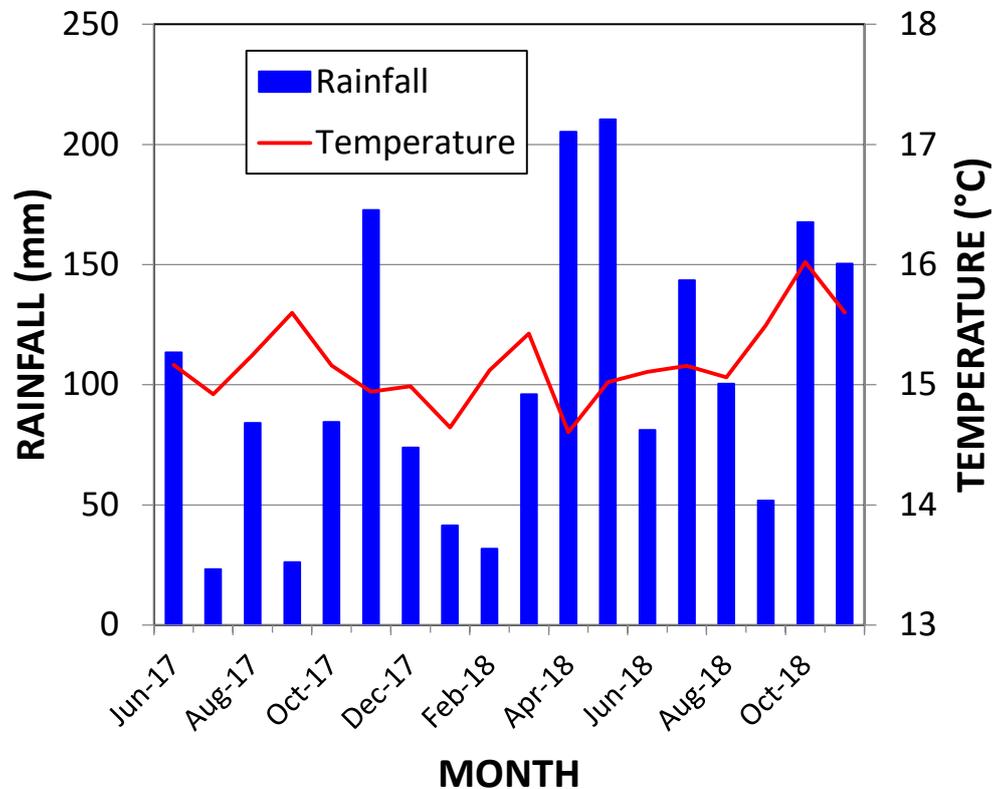


Figure 1: Average monthly temperature and rainfall from June 2017 to November 2018 at Kennedy meteorological station. (Data from the *Secretaria Distrital de Ambiente*)

While several human settlements living in the surroundings of *Bogotá* still have access to water through irregular connections to the public water network, tank cars, underground wells or rural aqueduct (Moreno and Garzón, 2018; Techo Colombia, 2015), most of *Bogotá* inhabitants have access in 2018 to drinking water supplied after treatment in one of the six drinking water production plants near the city: *Tibitoc* (production capacity: $10.5 \text{ m}^3 \cdot \text{s}^{-1}$), *Wiesner* ($14 \text{ m}^3 \cdot \text{s}^{-1}$), *La Laguna* ($0.4 \text{ m}^3 \cdot \text{s}^{-1}$), *El Dorado* ($1.6 \text{ m}^3 \cdot \text{s}^{-1}$), *Vitelma* ($1.5 \text{ m}^3 \cdot \text{s}^{-1}$) and *Yomasa* ($0.025 \text{ m}^3 \cdot \text{s}^{-1}$) (Jiménez Aldana and Santana López, 2017) (Figure 2). As *La Laguna* and *Vitelma* production plants are used only in contingency situations, the *El Dorado* production plant is the one which usually supplies drinking water to the *Usme* district; it operated below its capacity ($0.45 \text{ m}^3 \cdot \text{s}^{-1}$) (EAAB, 2019a). Its configuration is traditional with four groups of settling tanks-flocculators, two high flow stainless steel decanters, twelve filters with air-water wash, and chlorine and treated water contact tanks (EAAB, 2019a). The

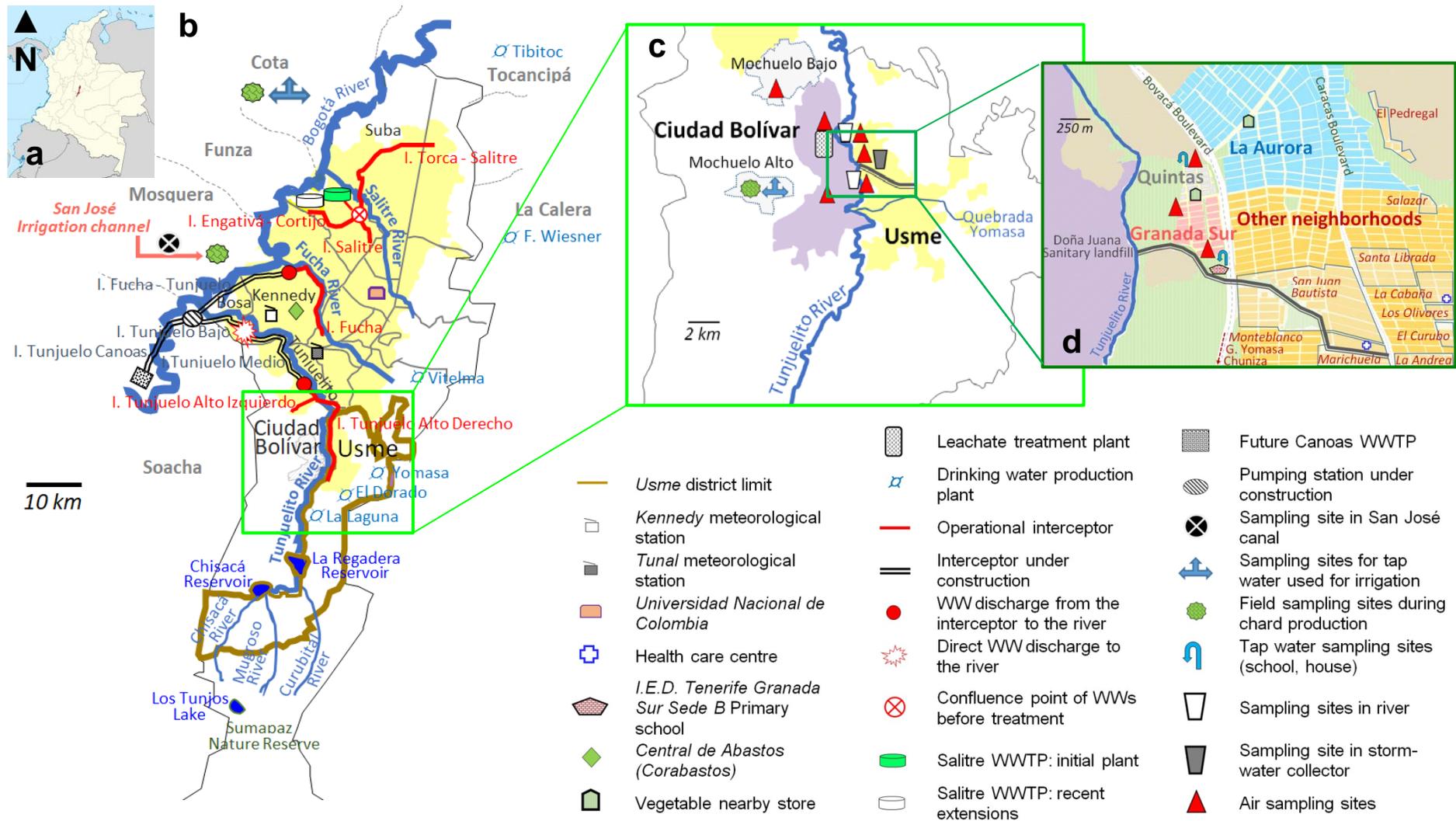


Figure 2a-d: map with location of sampling sites (a) at the Colombian scale, (b) at the Bogotá scale, (c) at the Usme scale, and (d) at the Granada Sur and Quintas del Plan Social neighbourhoods scale.

Vitelma, *La Laguna* and *El Dorado* production plants receive water via two pipelines from the *La Regardera* reservoir (Figure 2). Bogotá inhabitants consume about 0.1 m³ of water per person per day (Acosta, 2016; JICA, 2009). WWs are discharged into open air or buried interceptors that can be separate (e.g. those within the *Tunjuelito* catchment (Peña-Guzmán et al., 2017)) or combined with storm-water collectors (e.g. the canal *Cundinamarca* in the North-West of the *Fucha* catchment (Rojas et al., 2015)). Soiled toilet papers and diapers are collected independently and spread with other urban wastes on *Doña Juana* landfill surface, about 3 km to the west of the *Granada Sur* and *Quintas del Plan Social* neighbourhoods. The deposited wastes should be covered daily with clay, then ultimately with soil (UAESP, 2020); however, between 4 000 m² and 48 000 m² of wastes were not covered during the days of air sampling. While it was initially planned in the late 1980s to build for Bogotá a unique WWTP having a capacity of 22 m³.s⁻¹, the municipal authorities finally decided in the early 1990s to acquire three medium-sized WWTPs for the sub-watersheds of the *Tunjuelito*, *Fucha* and *Salitre* rivers in Bogotá (Costa Posada et al., 2015; Browder, 2009). A first WWTP in the northern part of Bogotá, *Salitre*, started its activities in 2000. Initially, it only carried out a primary treatment (screening, degreasing and chemically assisted particle settling). As it did not provide adequate treatment, a secondary activated sludge treatment unit and a disinfection unit with chlorine is currently under construction, with completion scheduled for September 2021 (Martínez et al., 2019; EAAB, 2019b; EAAB, 2018; CAR, 2009); its flow rate capacity would also increase from 4 to 7.1 m³s⁻¹. Currently, the future *Canoas* WWTP common for the *Fucha* and *Tunjuelito* sub-watersheds in Bogotá is under design (EAAB, 2018). The two main interceptors that will bring WW to this WWTP partially exist; they are currently discharging raw WWs into the *Fucha* river north of the *Kennedy* district, and into the *Tunjuelito* river southwest of the *Tunjuelito* district (Figure 2). In addition, some raw WWs are discharged directly through small interceptors in the *Bogotá* River. Some households are not yet connected to the sewer system and discharge their WWs elsewhere, e.g. into storm-water canals (Rojas et al., 2015); in 2018, this was the case of about 1835 households in *Usme*, many of them being in non-legalized neighbourhoods (EAAB, 2019a). The management of fresh water, drinking water, and WWs has been entrusted to the Public establishment *Empresa de Acueducto y Alcantarillado de Bogotá* (EAAB) from December 9, 1955 (Jaramillo-Giraldo and Rodríguez Villabona, 2004).

The commerce of fruits, vegetables, roots, tubers and beef is structured around similar supply chains in which smallholders predominate (Guarín, 2013). About 47% of the food items offered in the market place of Bogotá come from *Cundinamarca* department adjoining

Bogotá; through farmers' market or intermediaries (Eitzinger et al., 2014). Another 33% of the food passing through the *Corabastos* (*Central de Abastos*) wholesale market comes from the departments of *Boyacá*, *Tolima* and *Meta* (Eitzinger et al., 2014). Approximately 90% of the farmers in these departments are smallholders, producing roughly 70% of Bogotá's food, owning or cultivating less than 20 ha of land (Montoya and Mondragón, 2010). Chard is a minor vegetable crop: about 2300 tons produced from July 2017 to June 2018 (12 months) compared with for example 7400 tons of spinach, 97000 tons of lettuce and 791000 tons of tomatoes (data from the Database EVA of the *Ministerio de Agricultura y Desarrollo Rural*). However, it is a good model of leafy-vegetables that can be eaten raw in Colombia; Chards are cultivated all year round mainly in *La Sabana de Bogotá* (*Cundinamarca* Department), the duration of a culture being approximately 1.5 months.

Coarse estimates based on surveys of the farmers involved in our study show that about 55-75% and 5-35% of water inputs to chard crops result from irrigations during low and high rainfall months, respectively. Irrigation water is, for *Cota*, groundwater (also used as tap water) pumped in rural area near the city and then aerated to remove iron excess, filtrated on a mixed bed of sand and anthracite and disinfected with hypochlorite (CIDETER S.A.S., 2009), for *Mochuelo Alto* surface freshwater from *Quebrada El Chuscal* in rural area after its transport by an aqueduct, sand filtration and disinfection with hypochlorite (ESE Hospital Pablo VI Bosa, 2013), and for *Mosquera* water from a network of open canals in *Unidad 1* of the called *Sistema Hidráulico de manejo ambiental y contro de inundaciones La Ramada*, south of *Mosquera* and *Funza* (Figure 2). This network is fed by water pumped in *Bogotá* River at CAR *El Chicú* lifting station (0.6 to $2 \text{ m}^3 \cdot \text{s}^{-1}$) upstream the discharge of *Salitre* WWTP (CAR, 2011), by the runoff of rainwater to marshes, by the discharge into the marsh *Gualí – Tres Esquinas* of WWs from *Funza* (93 000 inhabitants (DANE, 2020b)) after secondary activated sludge treatment (Fiquitiva-Sierra and Bautista-Rojas, 2017), and in 2018 by the discharge of some of raw WWs of *Mosquera* as its WWTP started in late 2019. Excess of water in this network can be discharged into *Bogotá* River at lifting station *CAR El Tabaco* (maximum of $2 \text{ m}^3 \cdot \text{s}^{-1}$) downstream the confluence of the *Bogotá* River and its *Tunjuelito* tributary, at lifting station *CAR La Ramada* (maximum flow unknown) connecting *Guali – Tres Esquinas* march to *Bogotá* River between the confluences of the *Bogotá* River and its *Salitre* and *Fucha* tributaries, and at lifting station *CAR Mondoñedo* (maximum capacity of $1.25 \text{ m}^3 \cdot \text{s}^{-1}$ (Municipio de Mosquera, 2016; CAR, 2010)) that connects this network to *Bojacá* river. Upstream of lifting station *CAR El Chicú*, the *Bogotá* River received during our sampling period in 2018 WWs from *Chía* (132 000 inhabitants), *Cajicá* (82 000 inhabitants),

Tocancipá (39 000 inhabitants), *Gachancipá* (174 000 inhabitants), Suesca (17 000 inhabitants) and *Chocontá* (20 000 inhabitants) (DANE, 2020b) that were subject to pre-treatment (screening, particle settling) and secondary treatment (in oxidation ponds) (Mejía Puentes and Pérez Novoa, 2016; Méndez et al, 2011), as well as raw WWs from Villapinzón (17 000 inhabitants) (DANE, 2020b) and from Cota (32 000 inhabitants) (DANE, 2020b) where the WWTP was under construction during 2018 (EMSERCOTA, 2018). During low rainfall months, the typical flow of the *Bogotá* River is about $12 \text{ m}^3 \cdot \text{s}^{-1}$ before entering the city and $26 \text{ m}^3 \cdot \text{s}^{-1}$ downstream the confluence with *Tunjuelito* tributary (Energía de Bogotá, 2016; World Bank, 2012; SDA, 2008), while during high rainfall months the typical flow rate of the *Bogotá* River is about $34 \text{ m}^3 \cdot \text{s}^{-1}$ before entering the city and $84 \text{ m}^3 \cdot \text{s}^{-1}$ downstream the confluence with *Tunjuelito* tributary (SDA, 2008). Assuming 0.1 m^3 of WW per inhabitant per day, the typical flow rate of the *Bogotá* River enabled the dilution of pre-treated or raw WWs discharged upstream CAR *El Chicu* by 20 and 60 during low and high rainfall months, respectively. Similarly, the pumping flow of the *CAR El Chicu* lifting station apparently enabled the dilution of Funza WWs by a factor between 5 and 25, but its actual dilution depends on the actual water flows in the marsh *Tres Esquinas* and in the canals connected to this marsh. *Canals A, B, Tibaitatá, C* and *San José* supplied with water from this marsh are used to irrigate *Unidad 1* agricultural plots (CAR, 2010).

II.2. Experimental design

Water sampling: we monitored monthly between July 2017 and September 2018 the concentrations of *E. coli*, total coliforms other than *E. coli* (T.C.\E. coli), norovirus GI (NVGI), norovirus GII (NVGII), rotavirus type A (RV-A), *Salmonella* spp., *Shigella* spp., and *Cryptosporidium* spp. in the *Tunjuelito* River about 20 m upstream and 500 m downstream from the discharge point of a storm-water open collector, as well as near this point in this collector (Figure 2). The collector receives storm-water from *Chuniza, El Curubo, El Pedregal, Gran Yomasa, Granada Sur, La Andrea, La Aurora, La Cabaña, Los Olivares, Marichuela, Monteblanco, Salazar, San Juan Bautista* and *Santa Librada* neighbourhoods (corresponding to a total of around 84 000 inhabitants) and WWs from only 91 households according to (EAAB, 2019a). Water was sampled at 30-40 cm deep in water, 1-2 m and 3-3.8 m from the banks of the storm-water collector and the *Tunjuelito* River, respectively, using a 1.75 L low-density polyethylene beaker attached to a 3.8 m long stick. A first sample was used to measure *in situ* pH, temperature (°C), electrical conductivity ($\mu\text{S} \cdot \text{m}^{-1}$)

¹), and suspended solid ($\text{mg}\cdot\text{L}^{-1}$) of the water. A second sample (4 L) was stored in four 1 L low density polyethylene flasks for pathogen enumerations. From December 2017, a third sample and a fourth sample of 1 L each were stored in a 1 L low density polyethylene flask and a 1 L amber glass flask for characterizations of biological O_2 demand over 5 d (BOD_5) ($\text{mg}\ \text{O}_2\cdot\text{L}^{-1}$) and chemical O_2 demand (COD) ($\text{mg}\ \text{O}_2\cdot\text{L}^{-1}$), respectively. The flasks were transported at $0\text{-}4^\circ\text{C}$ to the *Universidad Nacional de Colombia* in *Bogotá*. The first four low density polyethylene flasks were stored in the *Laboratory of Molecular Virus Biology* at $0\text{-}4^\circ\text{C}$ until their use after 24h to 48h; the fifth low density polyethylene flask and the amber glass flask were stored in the *Environmental Engineering Laboratory* at $0\text{-}4^\circ\text{C}$ until their use after 24h to 72h. We monitored occasionally the concentrations of *E. coli*, T.C.\E. coli, NVGI, NVGII, RV-A, *Salmonella* spp., *Shigella* spp. and *Cryptosporidium* spp. in drinking water in the primary school *I.E.D. Tenerife Granada Sur Sede B* of the *Granada Sur* neighborhood, in a household of the *Quintas del Plan Social* neighborhood and in the Medicine Faculty of the *Universidad Nacional de Colombia* (*Bogotá* campus) (**Figure 2**): during low rainfall months (December 02, 2017; February 17 and 30, 2018) and high rainfall months (March 27, 2018; May 28, 2018). Water was sampled at the first tap between the water inlet and the storage tank, after disinfecting this tap and running the water for a few minutes ([Instituto Nacional de Salud, 2011](#)). *In situ*, a first 10 mL sample was used to measure residual chlorine in the water, and a second 200 mL sample was used to measure its pH, temperature, electrical conductivity and solids concentration. Then, 80 L to 100 L of water were transported in 120 L high-density polyethylene tanks with a flexible silicone hose to the *Laboratory of Molecular Virus Biology* and stored at room temperature, until use after 12h to 24h. We monitored occasionally the concentrations of *E. coli*, T.C.\E. coli, NVGI and shigella spp. in treated water from the landfill leachate treatment plant (**Figure 2**); water was sampled during high rainfall months (Mars 13, 2018; May 16, 2018) at 20 cm deep in the liquid reception tank using a 2 L low-density polyethylene beaker attached to a stick 2 m long, and at 2 cm deep of the leachate outlet channel of the plant, submerging a 0.5 L polyethylene beaker. 0.2 L of leachate was stored in one 0.25 L low density polyethylene flask that was transported at $0\text{-}4^\circ\text{C}$ to the *Laboratory of Molecular Virus Biology* and stored at $0\text{-}4^\circ\text{C}$ until their use after 24h to 72h. We monitored the concentrations of *E. coli*, T.C.\E. coli, NVGI, NVGII, *Salmonella* spp. and *Shigella* spp. in irrigation water; water was sampled during low rainfall months (December 4, 2017; January 30, 2018) and high rainfall months (Mars 11, 2018; August 06, 2018; October 31; 2018, November 23, 2018) in the *San José* canal supplying water to the rural areas of *Mosquera* at 10-20 cm deep and 1 m from the bank using a 1.75 L low-density polyethylene

beaker attached to a 2 m long stick, and at the hose connected to tap at the outlet of the drinking water networks in *Cota* (March 11, 2018; November 23, 2018) and *Mochuelo Alto* (August 6, 2018; October 31, 2018) (**Figure 2**), after running the water for a few minutes. Each sample (4 L) of water was stored in four 1 L low density polyethylene flasks, transported at 0-4°C to the *Laboratory of Molecular Virus Biology* and stored at 0-4°C until their use after 24h to 48h.

Air sampling: we monitored the concentrations of *E. coli*, T.C.\E. coli, and human enteric pathogens (NVGI, NVGII, *Salmonella* spp., *Shigella* spp.) in the air at a few dates (i) on the terraces of 3 houses in *Quintas del Plan Social* and *Granada Sur* neighborhoods around 7-10 m high above the streets, (ii) on the *Doña Juana* landfill about 3.3 km south-west, at the landfill leachate treatment plant about 600 m north-west, (iii) on the sites where water was sampled from the *Tunjuelito* River and from the storm-water canal around 1.8 m above the ground surface, (iv) on the terrace of a house in the *Mochuelo Bajo* area about 3 km south-west around 5 m above the street, and (v) on the campus of the *Universidad Nacional de Colombia* about 13.8 km north-east around 10 m above the ground surface (**Figure 2**). Air was sampled and biological particles were trapped in a phosphate buffered saline (PBS) at pH 7.4 by sampling stations that combined in series a 125 mL glass Impinger (AGI-30 7540-10, Air Sampling, ACE GLASS Incorporated, Vineland, NJ, USA) containing initially 50 mL of PBS, 1.5 m of sterilized silicone tubing, a rotameter (AALBORG P11A4, New York, NY, USA), 1.7 m of sterilized tubing and a vacuum pump (GAST DOA PT04 AA, Gast Manufacturing, Benton Harbor, MI, USA or WP6211560, Merck Millipore Sigma, Burlington, MA, USA) (**Photos 1a-d**). The air was pumped for 5h to 6h at 12.5 L.min⁻¹ and projected within/over the PBS, always leading to PBS bubbling allowing the trapping of all or part of the biological particles ([Han and Mainelis, 2012](#); [Hogan et al., 2005](#)). The Impinger with its residual PBS content was thereafter transported at 0-4°C to the *Laboratory of Molecular Virus Biology*, its openings being covered with sterile aluminum foil. For each sample, 14 to 40 mL of air-impacted PBS were immediately aliquoted in equal amounts into two polyethylene tubes. One of the tubes was immediately used to enumerate bacteria (see procedure in 2.3.); the other was stored at 4°C for up to 15 d to concentrate and enumerate viruses (see procedure in 2.3.).

Chard sampling: we monitored the quantities of *E. coli*, T.C.\E. coli, and human enteric pathogens (NVGI, NVGII, *Salmonella* spp., *Shigella* spp., *Cryptosporidium* spp) retained on



Photos 1a-d: Air sampling station used to trap airborne human pathogens: (a) entire air sampling station on the terrace of a house of *Quintas del Plan Social* neighborhood; (b) vacuum pump; (c) flow tube rotameter; and (d) Impinger with PBS.

the outer surface of chard leaves and petioles during production in municipality of *Cota*, in rural area of *Mochuelo Alto*, and in *San José* farm in *Unidad 1* of *Distrito de Riego de la Ramada y La Herrera* (Heliodoro and Bustos-López, 2018) (Figure 2 and Photos 1a-d). For chards produced in *San José* farm, contamination was also characterized just after harvest, after washing with water of irrigation canals during transport to *Corabastos* (*Central de Abastos*) wholesale market in *Bogotá*, after washing with tap water during storage in this wholesale market, and in two nearby stores (between *Granada Sur* and *Quintas del Plan Social* neighbourhoods, and in *La Aurora II* neighbourhood) upon arrival and about 6 or 21 h later after possible customer handlings. Chards were sampled during low-rainfall months (January to March 2018, or August 2018) and high-rainfall months (April to May 2018, or October 2018). Each sample consisted of 200-250 g and 200-250 g of fresh leaves and petioles for quantification of viruses and bacteria/unicellular parasite, respectively. During cultivation, the petioles of 10-12 leaves (in 10-12 plants) were cut 10 cm above the soil surface in a mini-plot of 12 m², 1 to 48 h after the last irrigation of the crop during low-rainfall and high-rainfall months. At harvest, bundles of approximately 10-12 leaves of chards

with their respective petioles were sampled on production sites from baskets receiving the harvested crop. During transport, chards were sampled in a truck transporting them to the wholesale market, 2-5 km after departure. At the wholesale market, chards were sampled directly in a large cellar in the market place. In the two nearby stores, chards were sampled just after their arrival between 9h30 and 11h to avoid customer handling, and at about 16h-17h30 the same day or at 8h the next morning on the merchant's stall after customer handling.



Photos 2a-d: water path between the *San José* canal and chard field near *Mosquera*: (a) *San José* canal in which water is pumped; (b) water pumping station; (c) field with young chards and a line of sprinklers; and (d) chard field near harvest.

II.3. Experimental procedures

Concentration and purification of biological materials in waters: for subsequent virus quantification, water samples were first clarified. For each sample, either 3 L from river, storm-water canal or irrigation water, or 0.1 L from the landfill lixivate diluted in 0.9 L of sterilized distilled water were pre-filtrated through a 90 mm paper filter having 8-12 μm pores (FTR 3.102.090, BOECO, Hamburg, Germany), while each 10 L sample of tap water from

the primary school, the household in *Quintas del Plan Social*, and the Medicine Faculty was pre-filtrated through a 90 mm polyamide filter having 0.2 μm pores (Sartorius 2500790G, Göttingen, Germany). The clarified samples from river, storm-water collector and irrigation waters were collected in three 1 L glass bottles, while landfill lixiviates were collected in a 1 L polypropylene beaker and the tap water samples were collected each into two 60 L high-density polyethylene tanks. The 8 to 10 filters used to clarify each tap water sample were immersed in 40 mL of PBS (pH 7.4) in two 50 mL flasks of low density polyethylene, and kept as such at 4°C for 6-8 hours until used for bacterial cultures, DNA extraction and qPCR test. Viruses were then concentrated and purified using methods adapted from [Haramoto et al. \(2004\)](#) for tap water and [Haramoto et al. \(2005\)](#) for other samples. Practically, viruses contained in the 3 L pre-filtrated from each sample from river, storm-water collector and irrigation water, or in the 0.1 L of each sample from landfill leachate were first immobilized on one 90 mm hydrophilic mixed cellulose ester filter having 0.45 μm pores (HAWP09000, MF-Millipore Sigma, Burlington, MA, USA) after being doped with Al^{3+} to give it a positive charge. Except for tap water, one filter only was used for each sample; it was doped with 5 mL of 250 mM AlCl_3 prior to filtration of each 500 mL of water. By contrast, one filter doped only once with 4 mL of 250 mM AlCl_3 was used to filter each 20 L of tap water. The viruses were then eluted from each filter by passing first 200 mL of a 0.5 mM H_2SO_4 solution at pH 3 so that the protons substituted for the Al^{3+} ions, then either 10 mL of a solution of 1 mM NaOH at pH 10.8 for the filters used for tap water, or 30 mL of the same solution for other water samples. Each of the 4 or 5 10 mL eluate samples for tap water was collected in a centrifuge tube which previously received 50 μL of 100 mM H_2SO_4 at pH 1 and 100 μL of 100-fold concentrated Tris-EDTA at pH 8 in order to neutralize the pH of the eluate; they were then pooled in a single 50 mL polypropylene tube. The other eluate samples of approximately 30 mL were collected in a 50 mL polypropylene centrifuge tube with a polyethylene cap having previously received 150 μL of 100 mM H_2SO_4 at pH 1 and 300 μL of 100-fold concentrated Tris-EDTA and pH 8. The eluates were supplemented with 1 g of polyethylene glycol 8000 (PEG 8000) powder per 10 mL of eluate. The mixtures were stirred gently at 4°C for 12h using an UltraRocker 1660709 rocking shaker (BIO-RAD, Hercules, CA, USA), then centrifuged in 50 mL Nalgene Oak Ridge tubes (ref. 3114-0050, Thermo Fisher Scientific, Waltham, MA, USA) at 4°C and 10000 rpm for 30 min using a centrifuge Sorvall LYNX 4000 (Thermo Fisher Scientific, Waltham, MA, USA). The pellets were resuspended in 2 mL of Tris-Base buffer at pH 8 (0.26 M Tris + 0.8 M NaCl). Each of the 2 mL suspension was concentrated one last time in a CENTRIPREP-YM-50 tube (Merck

Millipore Sigma, Burlington, MA, USA) by subjecting the tube to four cycles of centrifugation at 2500 rpm and 4°C during 10 min, 5 min, 5 min and 3 min in a KASAI UNIVERSAL 320 R centrifuge (Hettich, Tuttlingen, Germany) until the suspension was reduced to 260-460 µL. It was then stored at -20°C until use 3 days to 2 months later.

For subsequent bacteria and unicellular parasite enumerations and for the special cases of drinking tap water, the 8 to 10 filters immersed in 80 mL of PBS (pH 7.4) were stirred at 250 rpm during 30 min using a benchtop orbital shaker (MaxQ™ Mini 4450, Thermo Scientific, OH, US.A), and the eluate was collected in two 50 mL sterile flasks. Each of these tap water samples (about 80 mL) were filtrated through a 47 mm cellulose nitrate filter having 0.2 µm pores (Sartorius 11407-47-ACR, Göttingen, Germany), as well as each of other samples used either without dilution (100 mL for *Cota* and *Mochuelo Alto* irrigation waters) or after having been diluted to either one hundredth (0.2 mL for the *Tunjuelito* river upstream the storm-water canal mixed with 19.8 mL of sterilized distilled water) or to a thousandth (0.02 mL for the storm-water canal, the *Tunjuelito* river downstream this canal, the landfill lixiviates, and *Mosquera* irrigation water). The filters were immediately used for the enumerations of *E. coli* and T.C.\E. coli, then for the extraction of the DNA of pathogenic bacteria (*Salmonella* spp., *Shigella* spp.) and unicellular parasite (*Cryptosporidium* spp.) (see below).

Biological contamination of air: The aliquot of air-impacted PBS for subsequent virus GC enumeration underwent the same treatment as the viral suspensions recovered after elution from HA filters doped with Al³⁺, namely first a precipitation with PEG8000 followed by centrifugation and resuspension in a Tris-Base buffer, and then a concentration in a CENTRIPREP tube (see above). The aliquot of air-impacted PBS for subsequent bacteria and unicellular parasite enumerations was filtrated on through a 47 mm cellulose nitrate filter having 0.2 µm pores, the filter being immediately used thereafter for the enumerations of *E. coli* and T.C.\E. coli, and then for the extraction of the DNA of pathogenic bacteria (*Salmonella* spp., *Shigella* spp.) and unicellular parasite (*Cryptosporidium* spp.) (see below).

Elution and concentration of biological materials immobilised on chards: *E. coli*, T.C.\E. coli, NVGI, NVGII, *Salmonella* spp., *Shigella* spp. and *Cryptosporidium* spp. retained on the outer surface of chard leaves and petioles were eluted, concentrated and purified according to the following procedure. Viruses were first eluted from the external surface of 200-250 g fresh leaves and petioles of chards deposited on a disinfected 50 cm square of a

laboratory bench by wetting them, gently rubbing them with a 2 inch polyester nylon brush, and rinsing them with sterilized distilled water. Among the 1100 mL of water used, about 930-970 mL were collected, filtered through a low-density polyethylene strainer. Bacteria and unicellular parasites were eluted from the second sample of 200-250 g fresh leaves and petioles using the same procedure with a sterilized 0.154 M NaCl solution instead of water. Eluates for virus enumeration were then filtered onto 90 mm paper filters (FTR 3.102.090, BOECO, Hamburg, Germany) and stored each in a 1 L glass flask at 4°C until use 6h to 48h later. Eluates for bacteria and unicellular parasite enumeration were diluted 10-fold when they result from the washing of chards sampled during production, harvest or transport to the wholesale market. 50 mL of these eluates, 10-fold diluted or undiluted, were thereafter filtered through an sterile 47 mm cellulose nitrate filters having 0.2 µm pores, the filter being immediately used for the enumerations of *E. coli* and T.C.\E. coli, and then subsequently for the extraction of the DNA of pathogenic bacteria (*Salmonella* spp., *Shigella* spp.) and unicellular parasite (*Cryptosporidium* spp.) (see below).

Cultivable *E. coli* and T.C.\E. coli enumerations: Each 47 mm cellulose nitrate filters sterile used to filter water, PBS or plant wash water was deposited in sterile glass petri dishes, then received 2 mL of culture medium m-ColiBlue24 broth (HACH 2608450, Loveland, CO, USA) and incubated for 24h in a VO cool 400 incubator (Mettler, Schwabach, Germany) at 34°C and 5% CO₂. The colony forming units (CFU) of *E. coli* and T.C.\E. coli appeared then in blue and red, respectively (Grant, 1998; Grant, 1997), and were enumerated under a stereoscope (Ernst Leitz, Wetzlar, Germany). Filters were then stored at -20°C until used for *Salmonella* spp., *Shigella* spp. and *Cryptosporidium* spp. molecular analyses 2-8 months later.

Enumeration of genome copies of human enteric viruses: The viral RNA was extracted from concentrated and purified suspensions of viruses in CENTRIPREP-YM-50 tubes using the *PureLink™ Viral RNA/DNA Mini Kit* (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), according to the kit instructions, and the internal controls of the *Norovirus Genogroups 1 and 2 genesig Advanced Kit* and of *Human Rotavirus A genesig Advanced Kit* (Primerdesign™ Ltd, Eastleigh, UK).

To extract the viral RNA, first 200 µL of the concentrated and purified viral suspension at room temperature then 200 µL of the lysis buffer mixing L22 from *PureLink™ Viral RNA/DNA Mini Kit* (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA), 5.4 µL of the carrier RNA solution from *PureLink™ Viral RNA/DNA Mini Kit* and 3.7 µL of internal

control of extraction from the *Norovirus Genogroups 1 and 2 genesig Advanced Kit* or from *Human Rotavirus A genesig Advanced Kit* (Primerdesign™ Ltd, Eastleigh, UK) were put in a sterile 1.5 mL polyethylene centrifuge tube without RNase/DNase (80-1500 BIOLOGIX, St-Louis, MO, USA) having previously received 25 µL of Proteinase K solution from the *PureLink™ Viral RNA/DNA Mini Kit* to lyse viral particles. The tube was vortexed at 250 rpm for 15 s, then incubated at 56°C for 15 min in a VO cool 400 incubator, and finally centrifuged for 10 s at 500 rpm in a Microfuge 12 (Beckman Coulter, Brea, CA, USA) to remove solute drops on the tube lid and wall. After having added 250 µL of 99.8% ethanol, the tube was vortexed at 250 rpm for 15 s, incubated for 5 min at room temperature, and then centrifuged again for 10 s at 500 rpm. The lysate with ethanol (~675 µL) was transferred in a spin column within a 2 mL collection tube; the set was centrifuged at 7 000 rpm at room temperature for 1 min in the Microfuge 12. The viral RNAs adsorbed on the column which was then transferred to a second collection tube; 500 µL of a wash buffer containing ethanol and WII from *PureLink™ Viral RNA/DNA Mini Kit* was added at the top of the column, and the whole was centrifuged at 7 000 rpm at room temperature for 1 min. The filtered product was discarded and this washing operation was repeated a second time with 500 µL of the same buffer and the same collection tube. The spin column in a third 2 mL collection tube was centrifuged for 1 min at maximum speed (12 500 rpm) at room temperature to completely dry it. To eluate the viral RNA, the spin column was then placed in a 1.5 mL polyethylene centrifuge tube; 13 µL of sterile water without RNase were deposited at the top of the column which then incubated for 1 min at room temperature to remobilize the RNA; and the whole was then centrifuged at maximum speed for 1 min at room temperature. This elution procedure was repeated twice with final accumulation of approximately 39 µL of eluate in the 1.5 mL centrifuge tube. The spin column was then discarded and the 1.5 mL centrifugation tube with the purified RNA content was immediately covered and stored at -80°C until use.

Viral RNA was quantified using the *Norovirus Genogroups 1 and 2 genesig Advanced Kit* and the *Human Rotavirus A genesig Advanced Kit* (Primerdesign™ Ltd, Eastleigh, UK), as well as the *PrecisionPLUS OneStep RT-qPCR Master Mix* (Primerdesign™ Ltd, Eastleigh, UK). The targeted genes are listed in **Table 1**. In a first PCR cabinet with UV irradiation and additional down flow sterile air, 1 µL of one of the three primer and probe solutions for one of the three monitored viruses, 1 µL of the primer and probe solution corresponding to the internal extraction control, 3 µL of water without DNase/RNase and 10 µL of the Master Mix solution were put in a sterile 0.5 mL tube (ref. 80-0500 BIOLOGIX). After translation of the tube in a PCR cabinet with UV irradiation but without additional down flow sterile air, we

deposited in each tube 5 μ L of the purified RNA solution to be characterized or 5 μ L of one of the 7 dilutions of the three positive controls. The 20 μ L of each reaction mixture was then deposited in one of the wells of a 96-well Hard-Shell® PCR HSP9645 plates (Bio-Rad, Hercules, CA, USA), covered immediately with a MicroAmp™ Optical Adhesive Film (ThermoFisher Scientific, Waltham, MA, USA).

The plate was then inserted into a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA) programmed as follows: 10 min at 55°C for reverse transcription of viral RNA into cDNA, 2 min at 95°C for the activation of thermostable polymerase enzyme (iTaq polymerase) – antibodies which selectively inactivate the polymerase are irreversibly denatured after this hot period allowing this enzyme to function normally thereafter –, followed by 50 cycles with 10 s at 95°C for denaturation of all double stranded DNA then 60 s at 60 C for hybridization of the primers and TaqMan probe to gene of interest and elongation of the target DNA by DNA polymerase. The Real-Time PCR lasted a total of 1h55. PCR data was recorded and processed by Bio-Rad CFX Maestro software on Windows.

Pathogen	Target gene
Norovirus GI	Capsid protein gene
Norovirus GII	RNA dependent RNA polymerase gene
Rotavirus	Non structural protein 5 (NSP5) gene

Table 1: target genes in the RT-qPCR for viruses (norovirus GI, norovirus GII, rotavirus) and the qPCR for bacteria (*Salmonella* spp., *Shigella* spp. and unicellular parasites (*Cryptosporidium* spp.).

Enumeration of the genome copies of pathogenic bacteria and unicellular parasites: the DNA of *Salmonella* spp., *Shigella* spp. and *Cryptosporidium* spp. were pre-extracted from each of the 47 mm filters previously used for *E. coli* and T.C.\E. coli enumeration coming from the water, air and vegetable samples, after having cut them into small pieces of 1 to 5 mm² introduced into a sterile 2 mL polypropylene centrifuge tube (Corning Axygen MCT-200-C TF-20-R-S, NY, USA), by successively (i) disrupting the cells by putting 800 μ L of Tris-EDTA buffer (0.09 M Tris + 0.009 M EDTA, pH 8) in the tube and vortexing it for 5 min, (ii) precipitating the proteins by adding 800 μ L of a mixture of phenol/chloroform/isoamyl alcohol in the proportions of 4 mL/4.17 mL/1.834 mL and vortexing the tube for 2 min, then centrifuging it at 13 300 rpm for 20 min with a Heraeus™ Pico™ 17 microcentrifuge (Thermo Scientific, Waltham, MA, USA) and recovering 700 μ L

of supernatant in a second 2 mL tube, then repeating the procedure after adding 100 µL of Tris-EDTA to the tube with filter pieces, and (iii) precipitating the DNA in this second tube by adding 80 µL of 3 M C₂H₃NaO₂ equilibrated at pH 5.2 with HCl 0.1 M and 1200 µL of 99.8% ethanol, keeping the tubes at -80°C for 6h-8h to help precipitate DNA, then thawing their contents at room temperature for 3 to 5 min, centrifuging them at 13 300 rpm for 20 min, removing the supernatant, and resuspending the pellets in 200 µL of Tris-EDTA buffer. The tubes were then stored at 4°C until use in DNA extraction procedure 1h to 3h later.

Subsequently, The DNA in 200 µL of Tris-EDTA buffer were mixed with 400 µL of the lysis solution of the *Genomic DNA Purification Kit K0512* (ThermoFisher Scientific, Waltham, MA, USA) and 4 µL of the internal extraction control solution of the *Internal DNA Extraction Control Kit* (Primerdesign™ Ltd, Eastleigh, United Kingdom). The mixture was incubated at 50°C for 30 min in an ULE 500 incubator (Mettler, Schwabach, Germany) instead of at 65°C for 5 min as recommended in the K0512 kit instructions. The hydrophilic DNA was separated from the hydrophobic proteins by adding 600 µl of chloroform, vortexing the tubes at room temperature for 15-30 s, then centrifuging at 10 000 rpm for 20 min, instead of 2 min as recommended in the K0512 kit instructions. The aqueous phase above the chloroform containing the DNA was mixed with 800 µL of the DNA precipitation solution of the *K0512 Kit* in a 1.5 mL polyethylene centrifuge tube, vortexed for 30 s at 250 rpm at room temperature, and centrifuged at 10 000 rpm for 10 min, instead of 2 min as recommended in the *K0512 Kit* instructions. After having removed the supernatant, 100 µL of NaCl solution of the *K0512 Kit* were added to the pellet, and the tube was vortexed at 250 rpm for 1-2 min to ensure the complete dissolution of the DNA pellet. 300 µL of cold ethanol (stored at 4°C) were then added, and the DNA precipitated at -20°C during 4-8h, instead during the 10 min as recommended in the *K0512 Kit* instructions. The whole was then allowed to thaw for 1-2 min. To ensure the complete precipitation of the DNA, the tube was centrifuged at 10 000 rpm for 30 min (instead at 10 400 rpm for 3-4 min as proposed in the *K0512 Kit* instructions). After having removed the ethanol, the DNA pellet was dispersed in 50 µL of sterile deionized water (instead of 100 µL as proposed in the *K0512 Kit* instructions)). The concentrated and purified solutions of extracted DNA were stored at -20°C until use 1 to 15 days later.

DNA was quantified using *All pathogenic Salmonella species, Invasion protein (invA) gene – genesig standard kit* for *Salmonella* spp. (Primerdesign™ Ltd, Eastleigh, UK), *Shigella species, virulence plasmid pCP301 (VirA) – genesig standard kit* (Primerdesign™ Ltd, Eastleigh, UK) for *Shigella* spp., and *Cryptosporidium Oocyst wall protein (COWP) gene – genesig standard kit* (Primerdesign™ Ltd, Eastleigh, UK) for *Cryptosporidium* spp., an

Internal DNA extraction control kit (Primerdesign™ Ltd, Eastleigh, United Kingdom), and the *oasig*™ lyophilised 2X qPCR Master Mix (Primerdesign™ Ltd, Eastleigh, UK). In a first PCR cabinet with UV irradiation and additional down flow sterile air, 1 µL of one of the three primer and probe solutions for *Salmonella* spp., *Shigella* spp. and *Cryptosporidium* spp., 1 µL of the primer and probe solution corresponding to the *Internal DNA extraction control Kit*, 3 µL of water without DNase/RNase and 10 µL of the Master Mix solution were put in a sterile 0.5 mL tube (ref. 80-0500 BIOLOGIX). After translation of the tube in a PCR cabinet with UV irradiation but without additional down flow sterile air, we deposited in each tube 5 µL of the purified DNA solution to be characterized or 5 µL of one of the 7 dilutions of a of the three positive controls. The 20 µL of each reaction mixture was then deposited in one of the wells of a 96-well Hard-Shell® PCR HSP9645 plates, covered immediately with an MicroAmp™ Optical Adhesive Film.

The plate was then inserted into a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA) programmed as follows: 15 min of stabilization at 37°C, 2 min at 95°C for the activation of the polymerase enzyme (iTaq polymerase), followed by 50 cycles with 10 s at 95°C for denaturation of all double stranded DNA then 60 s at 60 C for hybridization of the primers and TaqMan probe to gene of interest and elongation of the target DNA by DNA polymerase. The Real-Time PCR lasted a total of 2h. PCR data was recorded and processed by Bio-Rad CFX Maestro software on Windows.

II.4. Access to other data

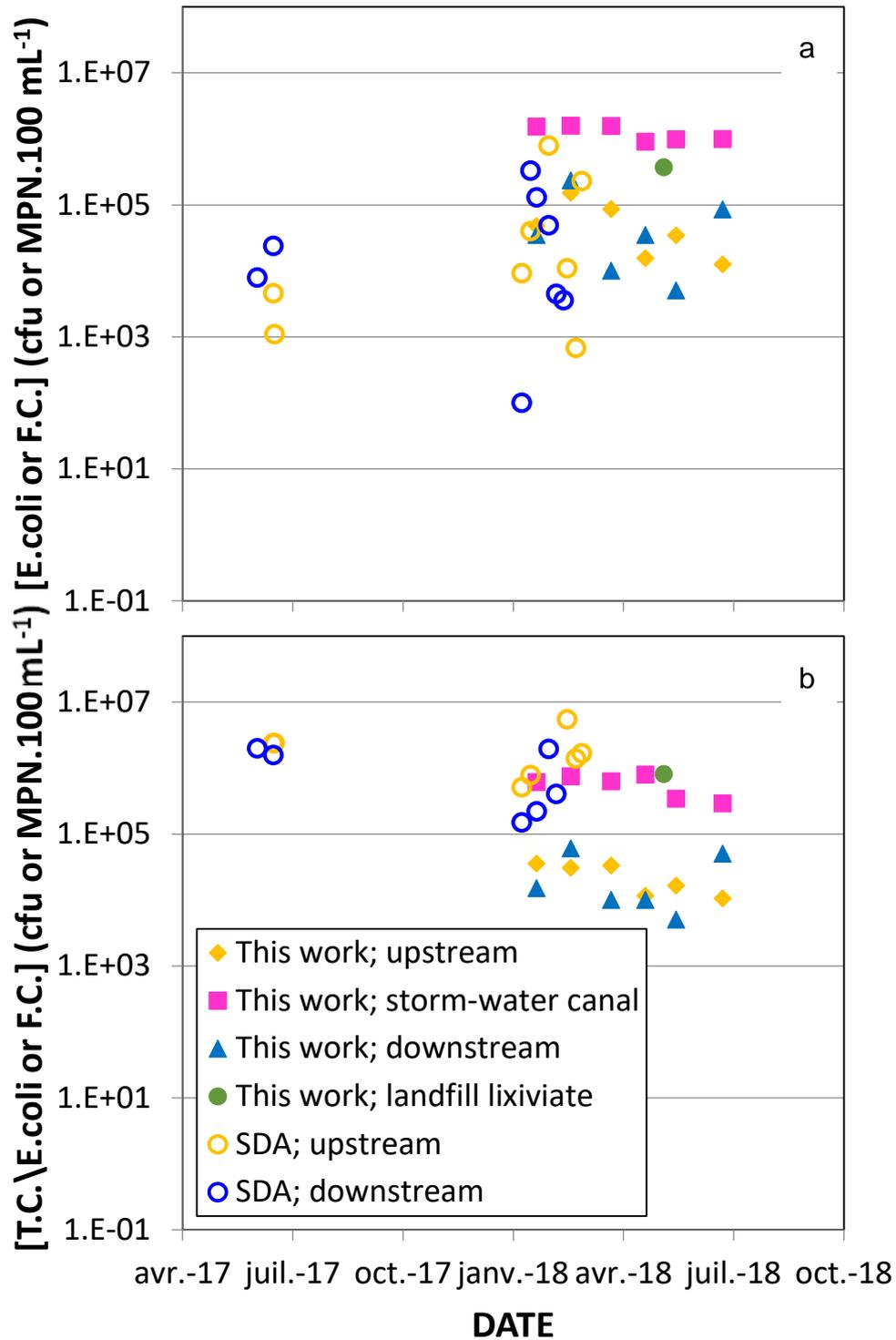
Climatic data from June 2017 to December 2018 were kindly supplied by the *Secretaria Distrital de Ambiente* (SDA) for all *Bogotá*'s meteorological stations, including *Kennedy* and *Tunal* ones, among others. Data include hourly wind direction (degrees) and speed ($\text{m}\cdot\text{s}^{-1}$), precipitation ($\text{mm H}_2\text{O}$) and temperature ($^{\circ}\text{C}$). Microbial contaminations characterized punctually over time of the *Bogotá* River, the irrigation canals, within the *Sistema Hidráulico de Manejo Ambiental y de Control de Inundaciones La Ramada*, and the marches in the same area by *E. coli* ($\text{MPN}\cdot 100 \text{ mL}^{-1}$) and T.C. ($\text{MPN}\cdot 100 \text{ mL}^{-1}$) were kindly supplied by the *Corporación Autónoma Regional* (CAR); a few additional data on T.C. and faecal coliforms (F.C.) in *Bogotá*, *Salitre*, *Fucha* and *Tunjuelito* rivers were kindly supplied by SDA ($\text{MPN}\cdot 100 \text{ mL}^{-1}$), and T.C. concentrations (in $\text{MPN}\cdot 100 \text{ mL}^{-1}$) only in raw and treated wastewater from Funza were kindly supplied by the town hall of Funza. The *Unidad Administrativa Especial de Servicios Públicos* (UAESP) of the City Council of *Bogotá* kindly

sent us the landfill waste areas not covered by clay or soil on the days when we sampled air.

III. Results and Discussion

III.1. Water contamination

In rivers, the concentrations of *E. coli* or F.C. and other coliforms (T.C.\E. coli or T.C.\F.C.) vary with location. On a 3 km section of the *Tunjuelito* river near *Usme* district that is bounded by two SDA sampling sites and that includes our sampling sites – that is, very much upstream of raw WW discharge from the *Fucha* and the *Tunjuelito* sub-watersheds – these concentrations did not vary greatly with location, even if this section of the *Tunjuelito* river receives more contaminated storm-water and lixiviates from the *Doña Juana* landfill (**Figures 3a-b**), probably because of their important dilution in the river. The upstream contamination of the *Tunjuelito* river probably resulted from numerous punctual WW discharges in this river or in its upstream tributaries (*Quebrada Yomasa, Quebrada Chuniza etc.*). The concentrations of T.C.\E. coli or T.C.\F.C. and *E. coli* of F.C. in the *Tunjuelito* river were about 1 to 10^{+3} times lower than their concentrations in the *Bogotá* river upstream of the WW discharge from the *Salitre* WWTP (concentrations between $2.4 \cdot 10^{+5}$ and $2.4 \cdot 10^{+6}$ MPN.100 mL⁻¹ with a median equal to $4.3 \cdot 10^{+5}$ MPN.100 mL⁻¹ concentrations), and about 10 to 10^{+5} times lower than their concentrations downstream from the confluence with the *Tunjuelito* tributary (concentrations between $7.6 \cdot 10^{+6}$ and $3.7 \cdot 10^{+7}$ MPN.100 mL⁻¹ with a median equal to $2.2 \cdot 10^{+7}$ MPN.100 mL⁻¹). The concentrations of *E. coli* and T.C.\E. coli in the *Bogotá* river were higher than currently observed in rivers around other big cities, e.g. in the *Thames* river near *London* ([Amirat et al., 2012](#)), the *Seine* river near *Paris* ([Mons et al., 2009](#)), the *Ebre* river near *Saragosse* ([Bouza-Deaño et al., 2008](#)) and the *Rio Grande* river near *Rio Grande City* ([LaWare and Rifai, 2006](#)), surely resulting simultaneously from the small flow of the *Bogotá* river, the huge population of *Bogotá* and surroundings and lack or insufficient treatment of wastewater in and around *Bogotá*. Upstream of the lifting station *CAR El Chicú*, the *Bogotá* river received during our sampling period in 2018 WWs from about 481 000 inhabitants (see subsection 2.1.). Between the two CAR sampling points on the *Bogotá* river, pre-treated WW was discharged from *Salitre* WWTP through the *Salitre* river, as well as raw WWs coming from the *Fucha* and the *Tunjuelito* sub-basins that are discharged in the *Tunjuelito* and *Fucha* tributaries near their confluence with the *Bogotá* river. Downstream of the SDA downstream



Figures 3a-b: Seasonal variations of [E. coli] and [F.C.] (a) and [T.C.\E. coli] and [T.C.\F.C.] (b) measured in the *Tunjuelito* river. (◆, ■, ▲, ●: [E. coli] and [T.C.\E. coli] measured in this work (cfu.100 mL⁻¹); ○, ○: SDA [F.C.] and [T.C.\F.C.] upstream (near the confluence with *Quebrada Yomasa* tributary) and downstream (downstream the discharge of landfill lixivate), respectively, (MPN.100 mL⁻¹)).

sampling point, the discharge of some other raw wastewater coming from the *Tunjuelito* sub-basin may further increase *Rio Bogotá* contamination. In the *Tunjuelito* River, in the stormwater collector and in the landfill leachate, human pathogens were rarely detected: *Shigella* spp. (6 positive samples among 22 samples), *Salmonella* spp. (1 positive samples among 18), NVGI (3 positive samples among 49 samples), NVGII (6 positive samples among 45 samples), RV-A (0 positive samples among 17 samples).

In tap water (primary school, household, Medicine Faculty), we never observed microbial contamination except for two samples in the primary school *I.E.D. Tenerife Granada Sur Sede B* in which [T.C.\E. coli] were $1.22 \cdot 10^{+2}$ and $1.21 \cdot 10^{+2}$ cfu/100 mL at February 17 and March 27, 2018, respectively. In addition, the irrigation water resources in the municipality of *Cota* and rural area of *Mochuelo Alto* come from the networks supplying tap drinking waters these cities; while no contamination was observed at *Cota*, contamination in *E. coli* and T.C.\E. coli was observed at *Mochuelo Alto* (**Table 2**).

Production site	<i>Cota</i>	<i>Mochuelo Alto</i>	<i>Mosquera</i>
<i>E. coli</i> in irrigation water during low-rainfall months (cfu.(100 mL) ⁻¹)	0	284	$1.3 \cdot 10^{+6}$
<i>E. coli</i> in irrigation water during high-rainfall months (cfu.(100 mL) ⁻¹)	0	245	-
<i>E. coli</i> on chard external surfaces during low-rainfall months (cfu.g f.m. ⁻¹)	12	2.2	149
<i>E. coli</i> on chard external surfaces during high-rainfall months (cfu.g f.m. ⁻¹)	0	14	170

Table 2: Comparison of *E. coli* concentrations in irrigation water and on chard external surface during production between sites around *Bogotá*, during low- and high-rainfall months.

The contamination of irrigation waters greatly varies with the area around *Bogotá*, with *a priori* no microbial contamination in the municipality of *Cota* (treated groundwater), a small contamination in *E. coli* and T.C.\E. coli in the rural area of *Mochuelo Alto* (treated surface water from a rural area), and a huge contamination in *E. coli* and T.C.\E. coli near *Mosquera* (surface water from rivers and marshes conveyed by irrigation canals, including San José canal, that received raw and treated wastewaters from several municipalities without any additional treatment before irrigation) (**Table 2**). Concerning surface waters of *La Ramada* area (including waters of the *Bogotá* river and *Balsillas* river (called in its upstream part *Subachoque* river), the high linear correlation between [T.C.\E. coli] and [E. coli] (**Figure 4**) suggests that non faecal coliforms developed with the discharge of WWs – especially their

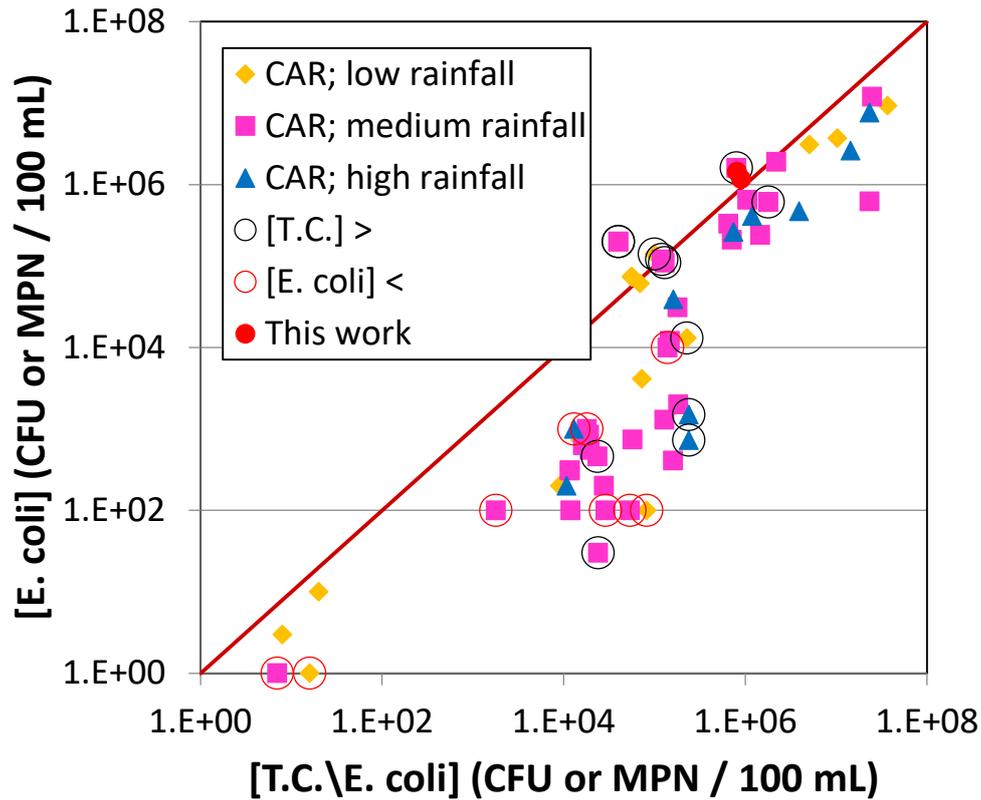
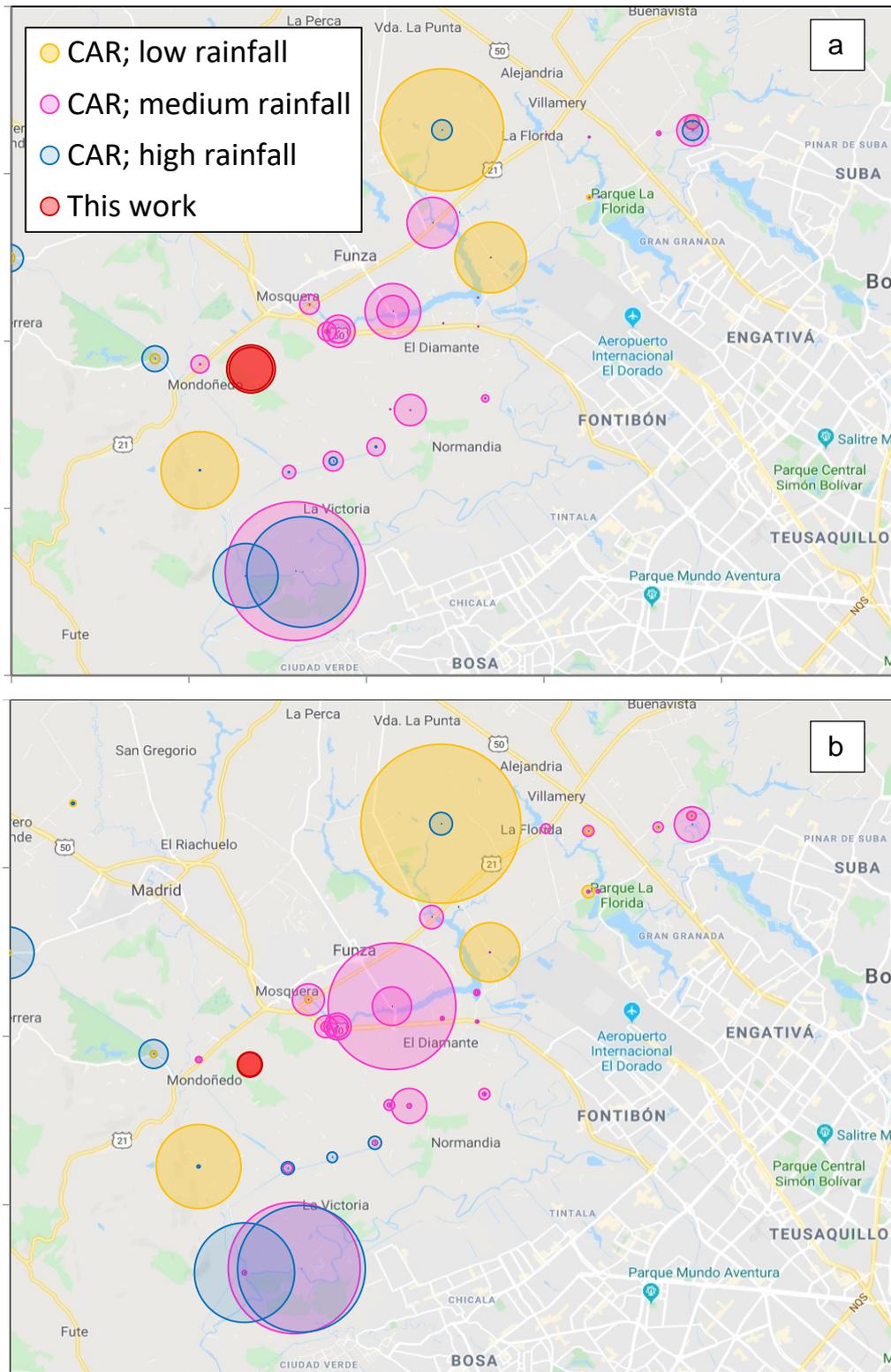


Figure 4: Relationship between [T.C.\E. coli] and [E. coli] concentrations in surface waters of *La Ramada* area during low rainfall months (◆) (< 70 mm.month⁻¹ just before), medium rainfall months (■) (70-140 mm.month⁻¹), and high rainfall months (▲) (> 140 mm.month⁻¹). Symbols surrounded by a red circle (○) or a black circle (○) corresponded to [E. coli] overestimated and [T.C.\E. coli] underestimated, respectively.

organic matter – in surface water bodies. We also note that high [T.C.\E. coli] values are associated with similar [E. coli] values, while low [T.C.\E. coli] values may be associated with nearly zero [E. coli] (Figure 4), as it may be the case for environmental waters without human or animal contaminations. There was no clear relationship between these values or their ratio and the abundance of rain in the past month (Figure 4). The spatial distributions of [E. coli] and [T.C.\E. coli] within *La Ramada* area varied considerably with the cumulative rainfall during the 30 days preceding each water sampling (Figure 5a-b). Such observation may result from the greatly varying contributions to *La Ramada* water resources of rainfall (and runoff water), *Bogotá* river, treated WW from *Funza* and (during our sampling) raw WW from *Mosquera*, to which are added at certain times the discharge of excess water to *Bogotá* and *Bojacá* rivers. The direction of the flow of water in this network of marshes and canals intended for irrigation can vary between periods, and lead to concentrating contamination of



Figures 5a-b: Measured [*E. coli*] (a) and [*T.C.\E. coli*] (b) in the surface waters of *La Ramada* area after low, medium and high cumulative rainfalls during the 30 days prior to sampling (i.e. <70 mm, 70-140 mm and >140 mm, respectively, during the 30 days preceding sampling). In this work, irrigation water was sampled twice during low rainfall months.

faecal origin in very different areas of this network. We note that the discharge of some of raw WWs of *Mosquera* until late 2019 in this irrigation network may have maximized the risk of faecal contamination of vegetables irrigated by water pumped in the San José canal.

III.2. Atmospheric contamination

E. coli and T.C.\E. coli were detected in about one fourth and one half, respectively, of the trapping solutions within impingers used to trap airborne microorganisms. More surprisingly, *Shigella* spp. was detected in about two third of the samples (**Table 3**). By contrast, *Salmonella* spp. was never detected, and NVGI and NVGII were detected in only a few samples.

	<i>E. coli</i>	T.C.\E. coli	<i>Shigella</i> spp.	<i>Salmonella</i> spp.	NVGI	NVGII
Positive sample frequency	23%	45%	50%	0%	2%	2%
Total sample number	65	65	60	18	58	54
Detect. threshold (cfu.m ⁻³ or GC.m ⁻³)	0.5	0.5	5	5	4	4

Table 3: Frequency of microorganism detection in air samples of about 4 m³. (Cryptosporidium spp. was sought, but not detected, in 3 air samples)

The following analysis focused on [*E. coli*], [T.C.\E. coli], and [*Shigella* spp.], the number of samples with positive concentrations being greater than 15 for each of them. We first tested whether the *Doña Juana* landfill site could be the main source of airborne microorganisms, by analysing airborne microorganism concentrations in different places according to the landfill waste areas not covered by clay or soil, their distance to the landfill site, the direction of the wind and its speed. None of the tested combinations allowed partly explaining these emissions, regardless of the microorganism considered. This strongly suggests that the contribution of the *Doña Juana* landfill to the aerosolization of airborne microorganisms was overall small; its negligible or small contribution to air contamination may have resulted from the small landfill waste area not covered by clay or soil, which varied between 0.4 ha and 5 ha for the days during which we sampled the air. The maps on which wind directions and speeds are reported in parallel to the concentrations of airborne microorganisms in air confirm the low contribution of the landfill site as wind directions corresponding to positive detection of airborne microorganisms greatly vary (**Figure 6a-c**). Moreover, nearly the same wind directions and speed may correspond to situations with or without airborne microorganisms.

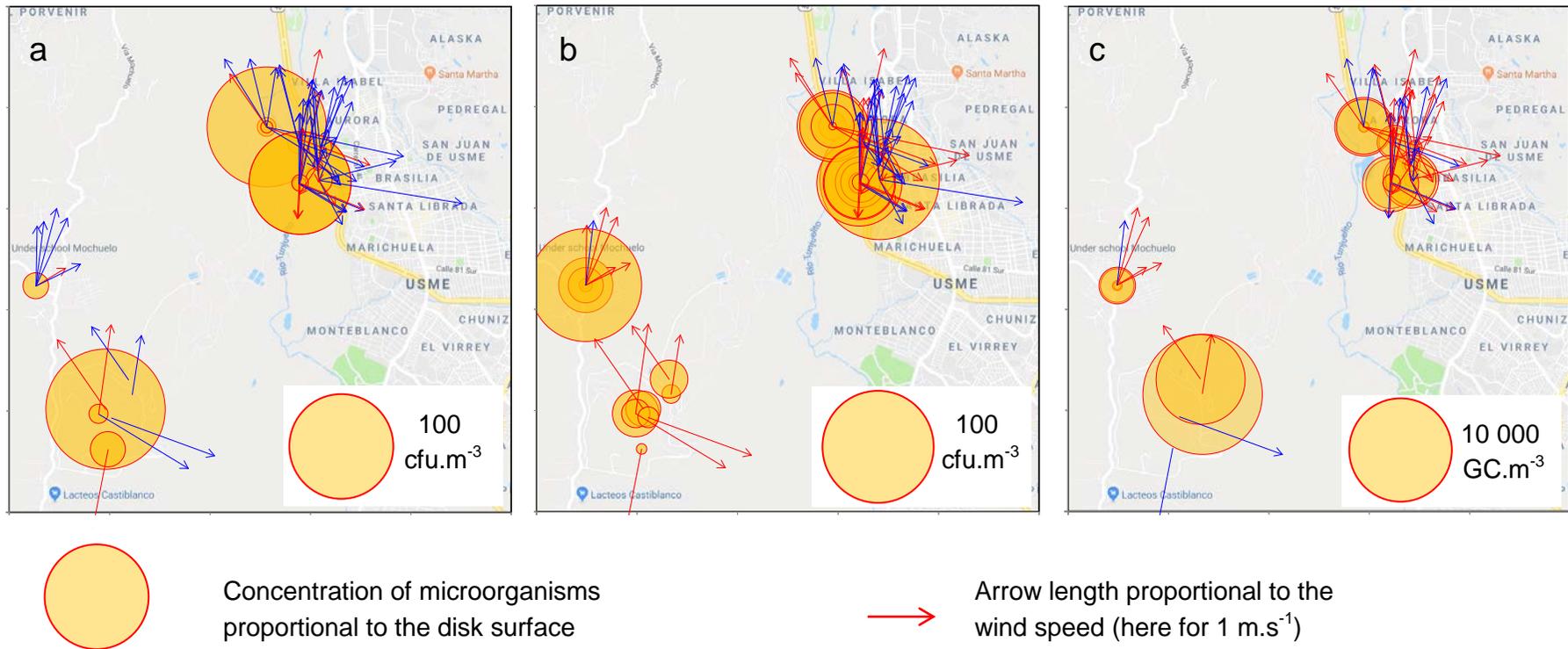


Figure 6a-c: Maps of airborne microorganisms at various dates in some sites for [*E. coli*] (a), [*T.C.E. coli*] (b), and [*Shigella spp.*] (c) with regard to the wind speed and direction. Red arrows correspond to cases where the considered microorganism was detected in air, while blue arrows correspond to cases without detecting it.

Considering alternatively southern winds (i.e. wind direction between 225° and 315°) and western winds (i.e. wind direction between 135° and 225°) could suggest that airborne microorganism aerosolization was higher during high-rainfall months than during low-rainfall months (Figure 7a-b). However, airborne microorganism concentrations seemed to be

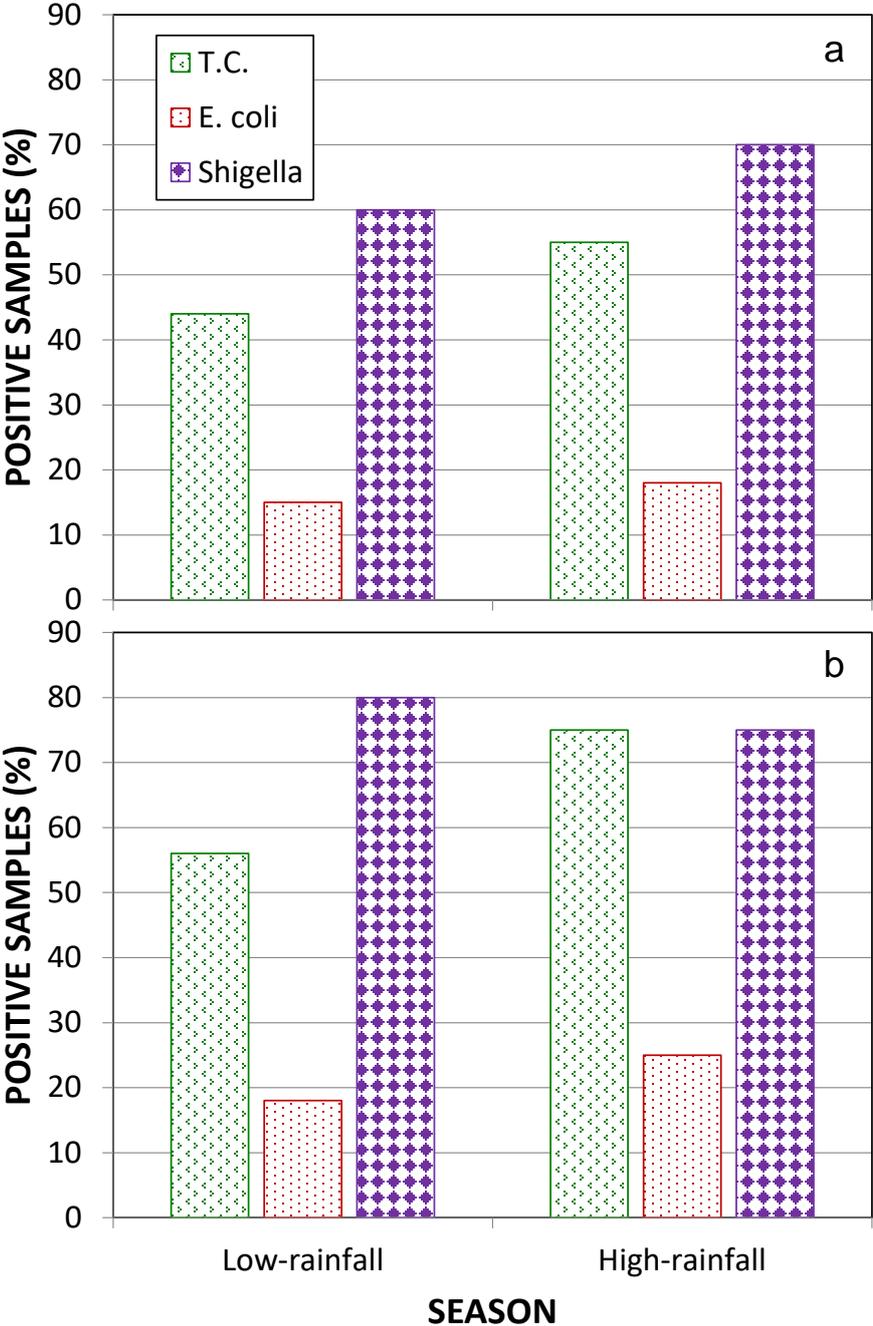


Figure 7a-b: Proportions of air samples in which T.C.\E. coli, E. Coli and Shigella spp. were detected during low-rainfall months (February-March 2018) and high-rainfall months (April-May 2018) for southern wind (i.e. between 225° and 315°) (a), and western wind (i.e. between 135° and 225°) (b).

negatively correlated with the rainfall amount between 2 d and 1 d before air sampling, suggesting that water may simultaneously reduce microorganism aerosolization from various interfaces with air and maintain their viability in air by increasing air relative humidity. Wind direction seems also to be an important criterion. Maximum [T.C.\E. coli] were observed for western winds. [E. coli] and [shigella spp.] were easily connected to the wind direction, although it seems that their maximum concentrations correspond to other wind directions, maybe northern winds for [E. coli] and southern winds for [shigella spp.]. The small range of wind speed variations (between 0.4 and 2.9 m.s⁻¹) and the correlations between wind speed on the one hand and rainfalls or wind orientation on the other hand may explain the lack of correlation between wind speed and airborne microorganisms in air.

All our attempts to adjust one of the concentrations in the air to the concentrations measured by functions taking into account the direction of the wind and the rains of the previous days in particular did not give any convincing result. Other variables should be monitored to have a chance to accurately describe these concentrations as the spatial and temporal distribution of emission sites, their surface moisture and the fate of airborne microorganisms in air.

III.3. Chard contamination at some steps between their production and consumption

During production, the contamination by *E. coli* of chards cultivated near *Mosquera* was high compared to the contamination of chards cultivated in the municipality of *Cota* and the rural area of *Mochuelo Alto*, without clear difference between low-rainfall and high-rainfall months (**Table 4**). The differences between the production sites surely resulted from the important contamination of irrigation water near *Mosquera* (**Table 2** and [Heliodoro and Bustos-López \(2018\)](#)) caused by the discharge of WWs in the *Rio Bogotá* upstream *CAR El Chicú* lifting station, the discharge of WWs from the municipality of *Funza* in the marsh *Tres Esquinas*, and the discharge in this irrigation network of raw WW from *Mosquera* until late in 2019.

E. coli contamination of chards cultivated near *Mosquera* during production, harvest and transport to the wholesale market were high, whatever the rainy period, compared to their contaminations during storage in this wholesale market and in two nearby stores, even though contamination seems to rise with exposure on the merchants' stall (**Figure 8a**). The no less significant, if not more severe, *E. coli* contamination of chard in production during the high-rainfall months (**Table 2** and **Figure 8a**) may have resulted from both the small number of samples, variations in chard maturity, time between sampling and last irrigation or rain, and

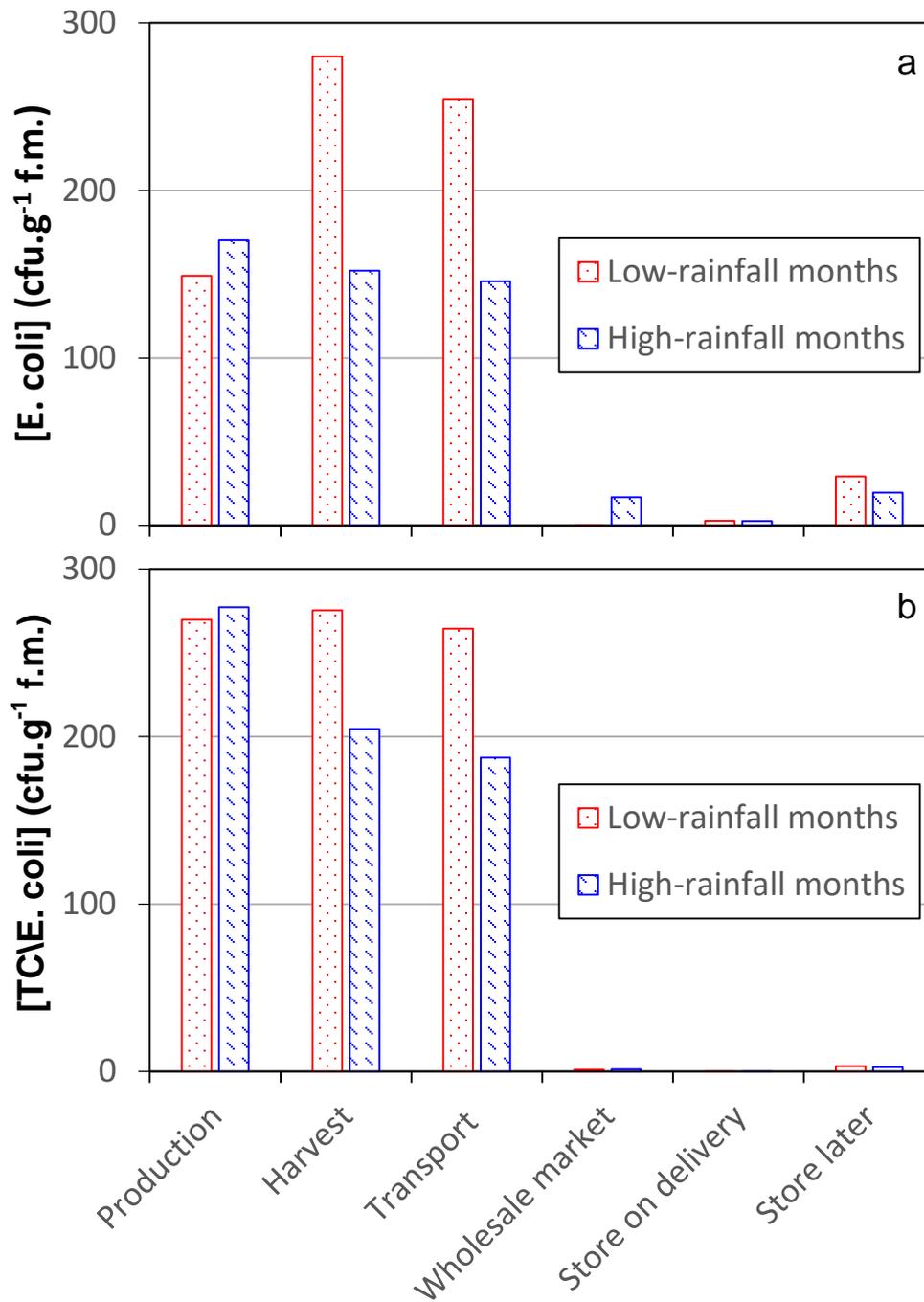


Figure 8a-b: Contamination by *E. coli* (a) and T.C. *E. coli* (b) of chards produced near *Mosquera* as a function of the steps between their production and consumption for samplings during low- and high-rainfall months.

the sampling location in the field. The *E. coli* contamination of chards cultivated during low-rainfall months was about twice the corresponding value for chards cultivated during high-rainfall months at harvest (i.e. 280 vs 152 cfu.g f.m.⁻¹) and during transport (i.e. 255 vs 146 cfu.g f.m.⁻¹) (**Figure 8a**): This ratio is consistent with the irrigation frequencies during the low-rainfall months and the high-rainfall months (approximately 4 vs 2 per week,

respectively). During low-rainfall months, the variability between sampling dates was low (variation coefficients of 21% and 24% at harvest and during transport, respectively), compared to the variability during production (54%), as the likely consequence of very localized field sampling during production, while bundles of approximately 10-12 leaves with their respective petioles from different chards were sampled just after harvest and during transport. During the high-rainfall months, the variabilities between sampling dates were high during production (64%), harvest (59%) and transport (78%); the highest contaminations were similar to those during the low-rainfall months, while the lowest contaminations corresponded to sampling of crops exposed to rainfall after their last irrigation. The lack of effect of washing at a standstill during chard transportation to *Bogotá* wholesale market *Central de Abastos* probably resulted from the use by the truck driver of water from San José irrigation canal. Conversely, washing at wholesale market was done with tap water and led to removals of *E. coli* and TC of more than 1-3.5 log₁₀. Such removals are consistent with observed values (Kinsinger et al., 2017) and values proposed in QMRA models (WHO, 2006; Amha et al., 2015). The increase in *E. coli* contamination on the merchants' stall was probably the consequence of customer handlings (Pérez-Rodríguez et al., 2019). Similar trends were observed for T.C.\E. coli with, however, fewer differences of contaminations between low-rainfall months and high-rainfall months, and almost no increase in contamination on the merchant's stall (Figure 8b), as T.C.\E. coli are also present in rainwater (Leong et al., 2017) and may better survive in the environment better than *E. coli* (Leclerc et al., 2001). T.C. concentrations are often 10 to 100 times higher than faecal coliforms (of which *E. coli* is the main representative). (e.g. Heliodoro and Bustos-López, 2018). The similarity between TC\E. coli and *E. coli* or faecal coliform concentrations in irrigation water near Mosquera and on chards during production and after harvest may have resulted from the high faecal contamination of irrigation waters that affect mainly faecal coliforms.

Chard contamination by *E. coli* is not the sum of the contamination from successive irrigations: immobilized *E. coli* can be mobilized and carried away by subsequent irrigations or rains (Lopez Giron, 2015) and decline when exposed to dry air conditions (Weller et al., 2017; Markland et al., 2013; Gutiérrez-Rodríguez et al., 2012). To assess the relationship between the contamination of irrigation water and the contamination of chards, we assumed that chards may trap a quantity of water during rain or irrigation proportional to its mass, as often proposed in quantitative microbial risk assessment (QMRA) models (e.g. 0.1 or 0.0178 g water.g⁻¹ f.m. for lettuce (WHO, 2006; Amha et al., 2015), 0.064 g water.g⁻¹ f.m. for cabbage (Amha et al., 2015), and 0.0036 g water.g⁻¹ f.m. for cucumber (Amha et al., 2015)).

Assuming between 0.01 and 0.03 g of water trapped per g of chard and considering only *E. coli* from the last irrigation, the ratio between *E. coli* concentration in chards (cfu.g⁻¹ chards) and in water (cfu.mL⁻¹) would then be comprised between 0.01 and 0.03. As an example, 250 cfu.g⁻¹ f.m. of chard would then result from irrigation by water having between 8 10⁺⁵ and 2.5 10⁺⁶ cfu of *E. coli* per 100 mL. Such values are consistent with our measures (**Table 2**) and with faecal coliform (F.C.) concentrations in the Canal *San José* variante *Mosquera* in 2004 (1.8 10⁺⁷ cfu.(100 mL)⁻¹), 2014 (4.5 10⁺³ cfu.(100 mL)⁻¹) and 2015 (1.0 10⁺⁷ cfu.(100 mL)⁻¹) ([Heliodoro and Bustos-López, 2018](#)).

We have tried to describe the contamination of chards by *E. coli* and its evolution between cultivation and transport to the wholesale market after their wash by the truck driver. *E. coli* extinction resulting from inactivation and death was described using the following equation:

$$\frac{\partial C(t)}{\partial t} = -k_{\text{ext}} \times C(t) \quad (1)$$

where $C(t)$ is the concentration of *E. coli* (cfu.g⁻¹ f.m.) at date t (h), and k_{ext} a coefficient of *E. coli* extinction (h⁻¹) that should depend on climatic conditions for *E. coli* on the external surface of leaves and petioles. Similarly, *E. coli* removal resulting from chard washing with water was described with the following equation:

$$\frac{\partial C(t)}{\partial W} = -k_{\text{wash}} \times C(t) \quad (2)$$

where W is the quantity of water watering the chards expressed in height of water (mm H₂O), and k_{wash} a coefficient of *E. coli* rinsing (mm⁻¹) that should depend on water temperature and physicochemical properties including its pH, mineral cations and organic solutes. We considered that rainfall, irrigation and water used to wash chards during their transport participate to *E. coli* removal. As the rainfall amount R and the irrigation amount I were expressed in mm H₂O, we substituted to the (unknown) quantity of water used to wash chards loaded in trucks during their transport to the wholesale market the height of water W_{tr} for a field watering bringing the same quantity of water to each chard plant. As irrigation and washing during transport used contaminated water from irrigation canals, they contributed simultaneously to chard contamination. Neglecting interactions between the extinction and the removal of *E. coli*, as well as variations in k_{ext} and k_{wash} , variations in chard contamination during a time interval Δt (fixed to 1h) may be expressed:

$$C(t + \Delta t) = (C(t) \times e^{-k_{\text{ext}} \times \Delta t} \times e^{-k_{\text{wash}} \times (R+I+W_{\text{tr}})}) + Q(I) + Q(W_{\text{tr}}) \quad (3)$$

where $Q(I)$ and $Q(W_{tr})$ are the quantities of *E. coli* retained by chards ($\text{cfu.g}^{-1} \text{ f.m.}$) supplied by irrigation and washing during transport, respectively, between t and $t+\Delta t$. Assuming that $Q(I)$ and $Q(W_{tr})$ did not vary with irrigation or washing events, we fit simulated chard contamination to measured contamination by estimating parameters $Q(I) = Q(W_{tr})$ and k_{wash} , that minimize the sum of the squares of the differences between simulated and measured concentrations for $I=W_{tr}=5.3 \text{ mm}$, and $k_{\text{ext}}=0.0035$ and by dividing the concentrations during production by 2 on the two dates when the samples were taken at the corner of the plot (i.e., January 3 and April 3, 2018). The correlation coefficient between simulated and measured *E. coli* concentrations was about 0.68 ($p>0.99$) (Figure 9).

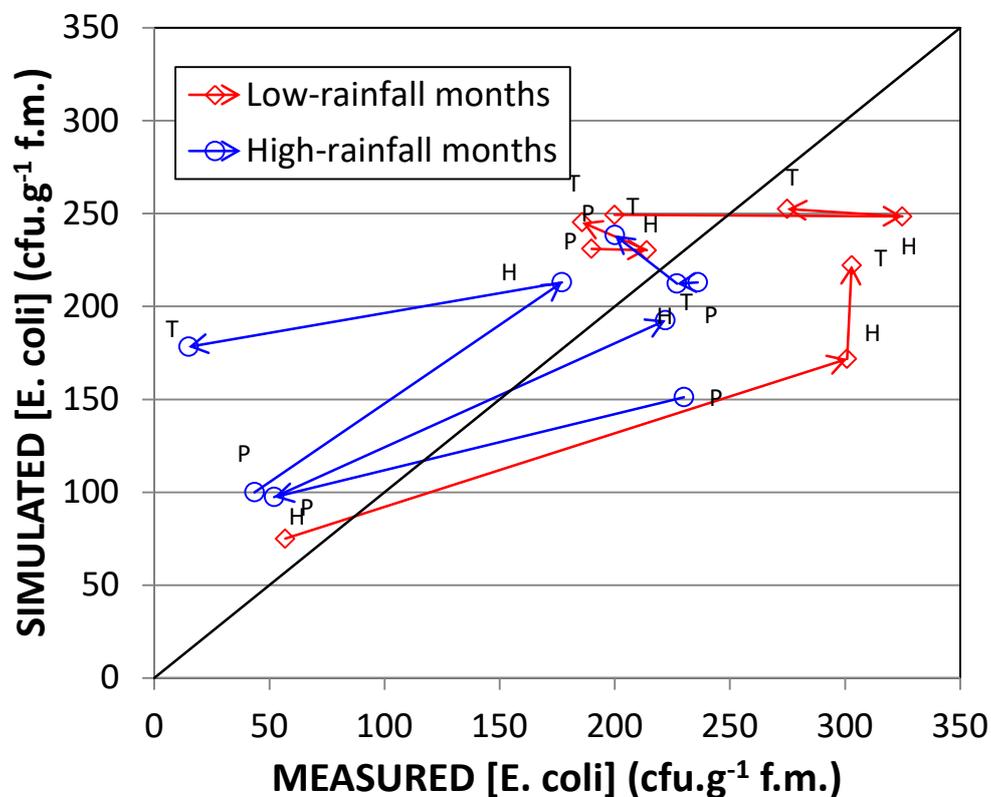


Figure 9: Simulated vs measured *E. coli* concentrations ($\text{cfu.g}^{-1} \text{ f.m.}$) on chards from *Mosquera* during production, at harvest and after washing during their transport to the wholesale market *Central de Abastos* of *Bogotá* using Equation (3), for $I=W_{tr}=5.3 \text{ mm}$, $k_{\text{ext}} = 0.0035 \text{ h}^{-1}$, $k_{\text{wash}} = 0.17 \text{ mm}^{-1}$, $Q(I) = Q(W_{tr}) = 154 \text{ cfu.g}^{-1} \text{ f.m.}$.

Among the pathogens sought on the surface of chard (NVGI, NVGII, *Shigella* spp, *Salmonella* spp., *Cryptosporidium* spp), only NVGI have been detected. Their concentration was then greater than or equal to $1.0 \cdot 10^{+0} \text{ GC.g}^{-1} \text{ f.m.}$ (Table 4). Failure to detect the same virus on the other samples indicates that it was absent or present at concentrations below the

detection threshold (approximately $0.1 \text{ GC}\cdot\text{g}^{-1} \text{ f.m.}$). As already mentioned in numerous articles, there was no correlation between the *E. coli* and NVGI concentrations (**Table 4**), faecal contamination being a necessary but not sufficient condition for contamination by enteric pathogens. In addition, the detection of NVGI in 3 samples (among 9) during the low-rainfall months, compared to the presence NVGI in 2 samples (among 9) during the high-rainfall months is compatible with more irrigation with contaminated water during the low-rainfall months, although probably not statistically significant.

	Low-rainfall months (January 30)	High-rainfall months (April 03)
Production	$7.1 \cdot 10^{+1}$ ($2.0 \cdot 10^{+2}$)	$9.7 \cdot 10^{+1}$ ($4.4 \cdot 10^{+1}$)
Harvest	$1.2 \cdot 10^{+1}$ ($3.0 \cdot 10^{+2}$)	- ($1.8 \cdot 10^{+2}$)
Transport	$8.1 \cdot 10^{+1}$ ($3.0 \cdot 10^{+2}$)	$1.0 \cdot 10^{+0}$ ($1.5 \cdot 10^{+1}$)

Table 4: Concentrations of NVGI on chard leaves and petioles ($\text{GC}\cdot\text{g}^{-1} \text{ f.m.}$) when they are above the detection threshold (of about $0.3\text{-}0.7 \text{ GC}\cdot\text{g}^{-1} \text{ fm}$), and in parenthesis corresponding concentrations of *E. coli* ($\text{cfu}\cdot\text{g}^{-1} \text{ f.m.}$).

IV. Conclusion

Faecal contamination mainly concerned fresh surface waters which received raw or insufficiently treated WWs. Such contaminations were mainly observed for the *Bogotá* river and *Tres Esquinas* marsh, which receives treated WW from *Funza* municipality; similar contaminations certainly affect the lower parts of two tributaries of the *Bogotá* river near their confluence with this river, the *Tunjuelito* and *Fucha* rivers which still receive raw WW from a large part of *Bogotá* city. Upstream in the *Tunjuelito* river, a lower contamination exist which can partly result from the illegal discharge of raw WW by some households not connected to the wastewater interceptors.

One of the major impacts of WW discharges is the contamination of irrigation water in the rural area of *La Ramada* (north-west of Bogota), the water coming partly from the *Bogotá* river and being transported by a network of canals linked to a few marshes including the *Tres Esquinas* marsh. In contrast, irrigation waters in the municipality of *Cota* and the rural area of

Mochuelo Alto were not or only slightly contaminated due to their origin (groundwater and fresh surface water from more rural area, respectively) and their treatment, because these waters were supplied simultaneously to households as drinking tap water and to farmers. During production, contamination of green leafy vegetables that can be eaten raw, such as chard, resulted from their irrigation with contaminated water. After harvest, their contamination could be increased by washing them with the same water. However, their subsequent washing with tap water at the main wholesale market in *Bogotá* removed 1 to 3.5 \log_{10} of *E. coli*, but the chard could again be contaminated on the merchant's stall following manipulations carried out by various customers. A model enabled to assess initial contamination of chards during production, at harvest and after washing before they arrived at the wholesale market; it would be easy to improve it by multiplying observations, and extend it to the last stages of the product's life before their purchase by end consumers by having indications on the practices of these customers in nearby stores, as well as on their hygiene.

E. coli and T.C.\ *E. coli* were detected in about one fourth and one half, respectively, of atmospheric air samples; their concentration in the atmosphere apparently depended on the direction of the wind and the rains of the previous days, without making it possible to identify the landfill as a privileged place of microorganism aerosolization.

It was not possible to find trends in human enteric pathogen variations, as they were not systematically found in water, air and vegetable samples. This may seem surprising for some viruses found almost systematically in surface water in other studies, but it can also be partly explained by the low volumes of water used in this study for these analyzes (3 L). Conversely, we were surprised to find shigella spp. in more than half of the air sample, without being able to explain it.

Currently, the *Bogotá* region is increasing its capacity to treat wastewater, in terms of volume and intensity of treatment, with the occasional addition of secondary - even tertiary - treatment, and one can imagine that from here a few years there will no longer be a major problem of faecal contamination of surface water.

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Chapter IV:

**Mouse intestinal villi
as a model system for studies
of Norovirus infection**

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Abstract

Noroviruses are enteric caliciviruses that have been detected in multiple species of mammals, including humans. Establishing an efficient *in vitro* cell culture system for human wild-type norovirus remains a challenge; however, replication of human norovirus 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 are a primary cellular model that has been shown to be permissible for the infection and replication of enteric viruses such as rotavirus, and the entire diversity of intestinal epithelial cells is found in the villi. We hypothesized that they could allow the infection and replication of the human wild norovirus. In this report we indicate that the isolated villi model of the mouse intestine is effective for the study of the infection and replication of the human wild norovirus from faeces and from environmental matrices (water, vegetables and air). To infect, the virus needs to be activated with trypsin and has an average replicative cycle of 10 h, although viral particles with infectious capacity are found from 2 h post-inoculation. The model is efficient in obtaining abundant biological material and is ideal for studying the biological activity of the human wild norovirus in the same cell model or for generating antibodies.

I. Introduction

Human noroviruses (HuNoVs) belong to the *Caliciviridae* family, they are single-stranded positive sense RNA viruses from 7.3 to 8.3 kb, their virions, unenveloped, are 38 to 40 nm in size (Robilotti et al., 2015) and are made by two structural proteins, VP1, which is organized into 90 dimers, is the main component of the capsid and VP2 of which only 6 copies are found per virion and is associated with VP1 on the surface internal (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).

HuNoV-related gastroenteritis is a self-limited disease characterized by diarrhea, vomiting, and general malaise (Rockx et al., 2002). It has been reported through histopathology that it affects the proximity of the small intestine presenting 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 HuNoVs replicate in immunocompetent individuals are still unknown. Recently, it was reported that HuNoVs replicated in intestinal enterocytes of transplant and immunocompromised patients, since the structural protein VP1 and non-structural antigens (RNA polymerase and viral genome-linked protein, VPg) were detected, providing direct evidence of viral replication in these cells (Karandikar et al., 2016). After the introduction of the rotavirus type A (RV-A) vaccine, HuNoV became the main cause of acute non-bacterial gastroenteritis affecting all age groups around the world but with special relevance in children <5 years, adults >65 years and immunocompromised persons (Kõivumägi et al., 2020; Morioka et al., 2017). Each year HuNoVs cause 64000 episodes of diarrhea requiring hospitalization being in turn responsible for 900000 severe infections of children in industrialized countries and more than 1 million infections and 200000 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 HuNoV Genogroup II.4 (O’Ryan, 2017), of the phylogenetic analysis proposed by Zheng et al. (2006). Transmission is typically through contaminated food, water, or environment, or person-to-person contact (Patel et al., 2009). However, the most common food vehicle for HuNoV remains ready-to-eat foods (Patel et al., 2009) and fresh produce such as vegetables and fruits (Callejón et al., 2015; DiCaprio et al., 2015; Berger et al., 2010), particularly those that require handling but no subsequent cooking (Widdowson et al., 2005). The study by Bennett et al. (2018) evaluating 972 epidemiological clusters associated with vegetables eaten raw in U.S.A. between the years 1998-2013 showed that the most commonly identified etiological agents were NoV (54%), Salmonella (21%) and shigatoxin-producing *E. coli* (STEC) (10%).

Despite their importance, the study and possible development of vaccines and treatments has been difficult, due to the lack of models for *in vitro* culture that allow their 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, HuNoV infection has been reported in B cells in a murine animal model, but this is not capable of recreating all aspects of norovirus infection (Oka et al., 2018; Jones et al., 2015; Taube et al., 2013). Efficient *in vitro* cell culture systems have been achieved for murine noroviruses 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 HuNoVs *in vitro* with high

levels of RNA (Ettayebi et al, 2016).

The study of murine norovirus has allowed understanding a little bit the biology of these viruses, it has been possible to determine a protein receptor CD300If and tropism towards cells of the tuft, a rare type of epithelial cells of the intestine that express the receptor (Wilen et.al, 2018). It has also been found to use M (microfold) cells to pass through the gastrointestinal epithelium without replicating in these cells (Gonzalez-Hernandez et al., 2013).

Isolated mouse intestinal villi is a primary cellular model that has been shown to be permissible for infection and replication of enteric viruses such as RV-A (Guerrero et al., 2010), has advantages by allowing the study in tissue cells where the virus naturally generates pathology, also because all the diversity of epithelial cells of the intestine is found, so it was hypothesized that it could allow infection and replication of HuNoVs. Therefore, this work sought to establish and identify the conditions for HuNoV infection in the mouse isolated intestinal villus model.

II. Materials and Methods

II.1. 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 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. Aliquot of diarrheal faeces was initially stored at -20°C and transported on ice to our laboratory for HuNoV studies. Thirty-seven faecal samples (34 children <5 years and 3 adults >75 years) were collected and the viruses were partially purified following the procedure of Guerrero et al. (2014). The partially purified viruses were kept at -20°C until it was used for immunodot-blot analysis, enzyme-linked immunosorbent assay (ELISA), RNA isolation, RT-qPCR or infectivity assay. The HuNoV antigen was detected in partially purified virus by immunodot-blot analysis by using anti-VP1 monoclonal antibody (Norwalk Virus (1D9), sc-53557 Santa Cruz, Biotechnology, INC) before detection with horseradish peroxidase (HRP)-conjugated bovine anti-mouse IgG secondary antibodies (sc-2371, Santa Cruz, Biotechnology, INC). Faeces samples were evaluated for parasites on microscopic examination, and the absence of other pathogens that cause diarrhea (RV-A, astrovirus

(AstV), *Salmonella* spp., *Shigella* spp. and *Cryptosporidium* spp. was verified with RT-qPCR and qPCR respectively, after isolation of the nucleic acids.

In addition, the presence of infectious HuNoVs was assessed on environmental samples collected during low, medium and high rainfall months (<70 mm.month⁻¹, 70-140 mm.month⁻¹ and >140 mm.month⁻¹, respectively) between July 27, 2017 and August 11, 2018 within and in the surroundings of *Bogotá* city (this region does not show great variations in temperature with the months). Practically, they included 7 air samples (i.e. 2 from two sites inside of the *Doña Juana* landfill, 1 from the landfill leachate treatment plant and 4 from terraces of houses around 7-10 m high above the streets and located approximately 3 Km north northeast of the landfill) with viruses of about 3.6-4.3 m³ air trapped in (initially) 50 mL of phosphate buffered saline (PBS) in 125 mL glass Impingers (AGI-30 7540-10, Air Sampling, ACE GLASS Incorporated, Vineland, NJ, USA), 32 water samples (i.e. 10 from the *Tunjuelito* river on its way through the *Usme* district of the *Bogotá* city, 10 from a storm-water open collector combined with wastewater that discharged its waters into this river, 2 from leachate treated in wastewater plant of the sanitary landfill, and 6 samples of drinking water from a primary school (3), a house (1) and the National University Campus(2) (located at 2.9 Km, 3.3 Km and 16.8 Km of the landfill, respectively), and 2 and 2 samples of irrigation water from a rural area of *La Ramada* near the municipality of *Mosquera* (at approximately 24 Km north-west of the landfill) and from *Cota* (at approximately 34 Km north-east of the landfill), respectively, with viruses first trapped on HA filters (HAWP09000, MF-Millipore Sigma, Burlington, MA, USA) doped with Al³⁺ after a prefiltration through 8-12 µm paper or 0.2 µm polyamide filters as described in **Chapter III** of the present manuscript; and 15 chard samples (leaves and petioles) sampled during the same months (including 2, 2 and 2 samples at rural area of *La Ramada* during cultivation, harvest and transport, respectively, 2 and 1 samples during cultivation at *Mochuelo Alto* rural area in *Usme* (at 1.3 Km of the landfill) and at *Cota*, 4 samples of the nearby stores of chard on a perimeter of 3.6 Km of the landfill, and 2 samples from *Corabastos* wholesale market in *Bogotá* at 15 Km of the landfill), with viruses eluted from external surfaces with sterilized water. After virus initial trapping and elution from samples of air, waters and fresh vegetables, viruses in solution were concentrated, purified and detected using immunodot-blot or PCR real time assays.

II.2. Partial virus purification

For the partial purification of the viruses present in the faeces, we followed the procedure of

[Guerrero et al. \(2014\)](#) with some modifications below. In a first sterile 1.5 mL centrifuge tube (réf. 80-1500 BIOLOGIX) and using a sterilized stainless steel spatula x150 mm, we put 0.1 g of the stool and dilute it in 0.6 mL of buffer Tris-Base at pH 8 (0.26 M Tris + 0.68 M NaCl) instead of 0.6 mL of TNC buffer at pH 7.5 (10 mM Tris, 150 mM NaCl, 1 mM MgCl₂, 10 mM CaCl₂). After having vortexed the tube for 1-2 min using BR-2000 1660610 (BIO-RAD, Hercules, CA, USA), we added 600 µL of 99.9% chloroform (instead of 1,1,2-trichlorotrifluoroethane used by [Guerrero et al. \(2014\)](#)) to precipitate proteins. The tube was then vortexed for about 5 min and subjected to 2 successive centrifugations at 13000 g for 10 min (instead of 1 performed by [Guerrero et al. \(2014\)](#)) using a HeraeusTMPicoTM 17 microcentrifuge (Thermo Scientific, Waltham, MA, USA). 580 µL of supernatant were collected in a second sterile 1.5 mL centrifuge tube. Then, 100 µL of Tris-Base buffer were added to the first tube and, after pellet dispersion and 2 centrifugations of 10 min at 13000 g, 100 µL of supernatant was recovered and added to the 580 µL initially removed. The viruses partially purified in 680 µL suspension were kept at -20°C (1-2 months) until used in RNA isolation procedure.

The partial purification of viruses from environmental samples varied with environmental matrices. Airborne viruses previously trapped in PBS were initially concentrated by mixing polyethylene glycol 8000 (PEG-8000) (9% w:v) to 10 mL PBS in 50 mL Nalgene Oak Ridge tubes (3114-0050, Thermo Fisher Scientific, Waltham, MA, USA), stirring gently the mixture at 4°C for 12 h using a rocking shaker (UltraRocker 1660709, BIO-RAD, Hercules, CA, USA) and then centrifuging the tube at 4°C and 10000 rpm for 30 min using a centrifuge Sorvall LYNX 4000 (Thermo Fisher Scientific, Waltham, MA, USA). The pellet was resuspended in 2 mL of Tris-Base buffer at pH 8 (0.26 M Tris + 0.8 M NaCl). Viruses coming from waters were concentrated using methods adapted from [Haramoto et al. \(2004\)](#) for tap water and [Haramoto et al. \(2005\)](#) for other samples (river water, storm water combined with wastewater, irrigation water, leachates and water produced from washing of chard-leaves surface). After immobilization on HA filters doped with Al³⁺, viruses were first eluted with 1 mM NaOH at pH 10.8, after having exchanged Al³⁺ with H₃O⁺ with H₂SO₄ at pH 3, and then concentrated using PEG-8000 and of Tris-Base buffer for concentration and resuspension respectively to 10-30 mL of product. Each of the 2 mL suspension was concentrated one last time in a CENTRIPREP-YM-50 tube (Merck Millipore Sigma, Burlington, MA, USA) by subjecting the tube to four cycles of centrifugation at 2500 rpm and 4°C during 10 min, 5 min, 5 min and 3 min in a KASAI UNIVERSAL 320 R centrifuge (Hettich, Tuttlingen, Germany) until the suspension was reduced to 260-460 µL. It was then

stored at -20°C until use 3 days to 2 months later.

II.3. RNA isolation

For viral RNA extraction from suspensions of the partial virus purified coming from faeces samples, we prepare initially in a tube (ref. 602052, Nest Scientific) 20 mL of disruptor buffer at pH 8 (including 0.26 M Tris, 0.09 M EDTA, 0.04 M SDS, 0.68 M NaCl and 1.3% β -mercaptoethanol 99%) to break the virus capsids. The disruptor buffer was saved at -20°C for a maximum of 8 days until its use, and just before the start of RNA extraction we added to each 20 μ L of this buffer and 4 μ L of internal control of extraction from the *Norovirus Genogroups 1 and 2 genesig Advanced Kit* or from *Human Rotavirus A genesig Advanced Kit* (Primerdesign™ Ltd, Eastleigh, UK). Then, the disruptor buffer with the internal extraction control was added to a sterile 1.5 mL centrifuge tube (ref. 80-1500 BIOLOGIX) containing 200 μ L of the viral suspension partially purified. Subsequently, to precipitate proteins and a pre-vortex to the tube (3000 rpm in BR-2000 1660610 per 2 min) we added 200 μ L of phenol 99.9% and given vortex newly for 2 min. Then, to remove the phenol, we added 200 μ L of 99.9% chloroform to the tube, given vortex for 2 min, centrifuged the tube at 12000 g for 8 min at room temperature (in Heraeus™Pico™ 17 microcentrifuge), recovered 200 μ L of the supernatant (using DLAB Scientific micropipettes Plus, ranges 10-100 μ L and 100-1000 μ L) and was placed in a second sterile 1.5 mL centrifuge tube (ref. 80-1500 BIOLOGIX). Then, we added to this second tube 200 μ L of 99.8% ethanol (cold at 4°C), vortexed it by about 30 s (at 3000 rpm) and allowed to precipitate for 12 h at -80°C. After these 12 h, we thawed the tube between 3-5 min and immediately centrifuged it at 13000 g per 12 min at room temperature to obtain a pellet. Subsequently, to dry this pellet, we eliminated the aqueous part gently turning the tube and using the *DLAB Scientific micropipette Plus* (range 10-100 μ L) and finally, we re-suspend the pellet in 50 μ L of deionized water and saved to -80°C until use (between 2-3 months).

On the other hand, to extract viral RNA of the partial suspensions coming from samples of air, river water, storm water combined with wastewater, irrigation water, leachates and water produced from washing of chard-leaves surface, we used the *PureLink™ Viral RNA/DNA Mini Kit* invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA) with some modifications. First in a sterile 1.5 mL polyethylene centrifuge tube without RNase/DNase (80-1500 BIOLOGIX, St-Louis, MO, USA), we add 25 μ L of Proteinase K solution from the *PureLink™ Viral RNA/DNA Mini Kit* to lyse viral particles, then 200 μ L of viral suspension at

room temperature, then 200 μL of the lysis buffer mixing L22 from *PureLink™ Viral RNA/DNA Mini Kit*, 5.4 μL of carrier RNA solution from the same Kit and 3.7 μL of internal control of extraction from the *Norovirus Genogroups 1 and 2 genesig Advanced Kit* or from *Human Rotavirus A genesig Advanced Kit*. The tube was vortexed at 250 rpm for 15 s, then incubated at 56°C for 15 min in a *VO cool 400 incubator* (Mettler, Schwabach, Germany), and finally centrifuged for 10 s at 500 rpm in a *Microfuge 12* (Beckman Coulter, Brea, CA, USA) to remove solute drops on the tube lid and wall. After having added 250 μL of 99.8% ethanol, the tube was vortexed at 250 rpm for 15 s, incubated for 5 min at room temperature, and then centrifuged again for 10 s at 500 rpm. The lysate with ethanol (~675 μL) was transferred in a spin column within a 2 mL collection tube; the set was centrifuged at 7000 rpm at room temperature for 1 min in the Microfuge 12. The viral RNAs adsorbed on the column was then transferred to a second collection tube: practically, 500 μL of a wash buffer containing ethanol and WII from *PureLink™ Viral RNA/DNA Mini Kit* was added at the top of the column, and the whole was centrifuged at 7000 rpm at room temperature for 1 min. The filtered product was discarded and this washing operation was repeated a second time with 500 μL of the same buffer and the same collection tube. The spin column in a third 2 mL collection tube was centrifuged for 1 min at maximum speed (12500 rpm) at room temperature to completely dry it. To eluate the viral RNA, the spin column was then placed in a 1.5 mL polyethylene centrifuge tube; 13 μL of sterile water without RNase were deposited at the top of the column which then incubated for 1 min at room temperature to remobilize the RNA; and the whole was then centrifuged at maximum speed for 1 min at room temperature. This elution procedure was repeated twice with final accumulation of approximately 39 μL of eluate in the 1.5 mL centrifuge tube. The spin column was then discarded and the 1.5 mL centrifugation tube with the purified RNA content was immediately covered and stored at -80°C until use 20 days to 2 months later in RT-qPCR and cellular infection.

II.4. Immuno-dot blot assay

20 μL of the partially purified virus suspensions were separately added onto polyvinylidene fluoride (PVDF) membranes and incubated for 1 h at 37°C. The membranes were blocked with 5% skimmed milk in PBS for 1 h at 37°C under continuous agitation. Dot blots were washed three times with PBS under continuous agitation and then incubated with 0.2 $\mu\text{g}\cdot\text{mL}^{-1}$ of anti-VP1 monoclonal antibody (Norwalk Virus (1D9), sc-53557 Santa Cruz, Biotechnology, INC) or 0.2 $\mu\text{g}\cdot\text{mL}^{-1}$ of monoclonal anti-rotavirus antibody (SC53560, Santa

Cruz Biotechnology) or polyclonal rabbit anti-astrovirus antibody respectively, in PBS containing 0.1% bovine serum albumin (BSA) for 1 h at 37°C. After washing three times with PBS, secondary HRP-conjugated goat anti-mouse antibody ($0.4 \mu\text{g.mL}^{-1}$, SC 2005, Santa Cruz Biotechnology Inc.) or HRP-conjugated goat anti-rabbit secondary antibody ($0.4 \mu\text{g.mL}^{-1}$, sc-2004, Santa Cruz Biotechnology Inc) were added for 1 h at 37°C. Peroxidase activity was revealed with aminoethylcarbazole substrate (Sigma, St. Louis, MO, USA) (0.64 mg.mL^{-1}) (from 4 mg.mL^{-1} stock solution in dimethylformamide, Sigma, St. Louis, MO, USA) in acetate buffer (30 mM sodium acetate, 12 mM acetic acid, pH 5.5) and 0.36% hydrogen peroxide (Sigma, St. Louis, MO, USA). Faeces samples and samples collected during low, medium and high rainfall months from river water, storm water combined with wastewater, irrigation water, leachates, air and chard-leaves samples were analyzed with this technique.

II.5. RT-PCR real time

Viral RNA was detected using the *Norovirus Genogroups 1 and 2 genesig Advanced Kit* and the *Human Rotavirus A genesig Advanced Kit* (Primerdesign™ Ltd, Eastleigh, UK) according to the instructions of these kits, preparing before several solutions (primers and probes, positive controls and Master Mix). In this kits each pair of primer and probe for HuNoV GI (NVGI) or HuNoV GII (NVGII) or rotavirus A (RV-A), was contained in lyophilized form in a 2 mL polypropylene tube respectively, and they were dissolved in 165 μL of RNase/DNase-free water. On the other hand, the solutions of positive controls corresponding to the three sequences of the genes to be amplified NVGI, NVGII and RV-A respectively were resuspended in 500 μL of template preparation buffer supplied in each kit, and all these several solutions were stored at -20°C until use. The Master Mix for RT-qPCR was prepared from a lyophilized Master Mix contained in a glass bottle of about 1.5 mL ref. *PrecisionPLUS OneStep RT-qPCR Master Mix* (Primerdesign™ Ltd, Eastleigh, UK) composed of dNTPs, MgCl_2 and stabilizers, by putting the latter in solution in the same flask in 525 μL of resuspension buffer present in the kit. Once the suspension was made it was stored at -20°C until used. Subsequently, we prepared the reactive mixtures by depositing in sterile 0.5 ml tubes (ref. 80-0500 BIOLOGIX) 10 μL of the Master Mix solution, 1 μL of one of the three primer and probe solutions corresponding to the virus to be quantified, 1 μL of the primer and probe solution corresponding to the internal extraction control, 3 μL of water without DNase or RNase and 5 μL of the purified RNA solution to characterize. The 20 μL of each reaction mixture was then deposited in one of the 96 wells of a PCR plate (Hard-Shell® 96-Well PCR

Plates, low profile, thin wall, skirted, green / white hsp9645) and covered immediately after filling with an optical adhesive film (4311971 *MicroAmp*[™] *Optical Adhesive Film*, (Thermo Fisher Scientific, Waltham, MA, USA). The plate was then inserted into CFX96 Touch[™] Real-Time PCR Detection System (BIO-RAD, Hercules, USA) programmed as follows: 10 min at 55°C for reverse transcription of viral RNA into cDNA, 2 min at 95°C for the activation of Tac polymerase enzyme (iTaq polymerase, mediated by high-temperature activation antibody), followed by 50 cycles with 10 s at 95°C for denaturation of the cDNA chains then 60 s at 60°C for hybridization of primers and *TacMan* sonde and collection data. The quantitative PCR lasted a total of 1 h 55 min. The qPCR data were recorded and processed by the Bio-Rad CFX Maestro software under Windows. With this technique were analyzed the stool samples and the environmental samples collected in low, medium and high rainfall months.

II.6. Partial DNA purification

For the partial purification of bacterial DNA (*Salmonella* spp., *Shigella* spp.) and parasite DNA (*Cryptosporidium* spp.) of faeces samples, we used the phenol-chloroform, by placing initially in a first sterile centrifuge tube of 1.5 mL (ref. 80-1500 BIOLOGIX) 0.1 g of stools to dilute them in 600 µL of Tris-EDTA buffer (0.09 M Tris + 0.009 M EDTA, pH 8), then we apply vortex to the tube for 5 min to promote cell detachment. Subsequently, we performed the precipitation of proteins by adding to this tube 600 µL of a mixture of phenol/chloroform/isoamyl alcohol in the proportions of 4 mL/4.17 mL/1.834 mL and vortexing the tube for 2 min to homogenize its content, then centrifuging it at 13000 g for 20 min with the Heraeus[™] Pico[™] 17 and recovering 580 µL of supernatant in a second 2 mL centrifuge tube, then repeating the procedure after adding 100 µL of Tris-EDTA to the tube with the stools and precipitating the DNA in the second tube by adding 68 µL of 3 M C₂H₃NaO₂ equilibrated at pH 5.2 with HCl 0.1 M. After, 1200 µL of 99.8% ethanol were added to the tube and it was keeping at -80°C for 6 h-8 h to help precipitate DNA, then thawing their contents at room temperature for 3 to 5 min, centrifuging it at 13000 g for 20 min, removing the supernatant, and resuspending the pellets in 200 µL of Tris-EDTA buffer. Each tube with the partially purified DNA was stored at 4°C until use in DNA extraction procedure 1 h to 3 h later.

II.7. DNA isolation

The DNA partially purified and suspended in 200 μ L of Tris-EDTA buffer was isolated mixing 400 μ L of the lysis solution of the *Genomic DNA Purification Kit K0512* (ThermoFisher Scientific, Waltham, MA, USA) and 4 μ L of the internal extraction control solution of the *Internal DNA Extraction Control Kit* (Primerdesign™ Ltd, Eastleigh, United Kingdom). The mixture was incubated at 50°C for 15 min in an *ULE 500 incubator* (Mettler, Schwabach, Germany) instead of at 65°C for 5 min as recommended in the K0512 kit instructions. The hydrophilic DNA was separated from the hydrophobic proteins by adding 600 μ L of chloroform, vortexing the tubes at room temperature for 15-30 s, then centrifuging at 10000 g for 20 min, instead of 2 min as recommended in the K0512 kit instructions. The aqueous phase above the chloroform containing the DNA was mixed with 800 μ L of the DNA precipitation solution of the K0512 Kit in a 1.5 mL polyethylene centrifuge tube, vortexed for 30 s at 250 rpm at room temperature, and centrifuged at 10000 g for 10 min, instead of 2 min as recommended in the K0512 Kit instructions. After having removed the supernatant, 100 μ L of NaCl solution of the K0512 Kit were added to the pellet, and the tube was vortexed at 250 rpm for 1-2 min to ensure the complete dissolution of the DNA pellet. 300 μ L of cold ethanol (stored at 4°C) were then added, and the DNA precipitated at -20°C during 4-8h, instead during the 10 min as recommended in the K0512 Kit instructions. The whole was then allowed to thaw for 1-2 min. To ensure the complete precipitation of the DNA, the tube was centrifuged at 10000 g for 30 min (instead at 10400 g for 3-4 min as proposed in the K0512 Kit instructions). After having removed the ethanol, the DNA pellet was dispersed in 50 μ L of sterile deionized water (instead of 100 μ L as proposed in the K0512 Kit instructions). The concentrated and purified solutions of extracted DNA were stored at -20°C until use in qPCR tests 1 to 15 days later.

II.8. qPCR assays

DNA isolated from stool samples was used for detection of *Salmonella* spp., *Shigella* spp. and the parasite *Cryptosporidium* spp., following the manufacturer's instructions of three quantification kits respectively: (i) *All pathogenic Salmonella species, Invasion protein (invA) gene – genesig standard kit* (Primerdesign™ Ltd, Eastleigh, UK); (ii) *Shigella species, virulence plasmid pCP301 (VirA) – genesig standard kit* (Primerdesign™ Ltd, Eastleigh, UK), and (iii) *Cryptosporidium Oocyst wall protein (COWP) gene – genesig standard kit*

(Primerdesign™ Ltd, Eastleigh, UK), using during the amplification *Internal DNA extraction control kit* (Primerdesign™ Ltd, Eastleigh, United Kingdom) and the oasig™ lyophilised 2X qPCR Master Mix (Primerdesign™ Ltd, Eastleigh, UK). The reaction mixture was carried out adding in a sterile 0.5 mL tube (ref. 80-0500 BIOLOGIX) 1 µL of one of the three primer and probe solutions for *salmonella* spp., *shigella* spp. or *Cryptosporidium* spp. respectively, 1 µL of the primer and probe solution corresponding to the Internal DNA extraction control Kit, 3 µL of water without DNase/RNase, 10 µL of the Master Mix solution and 5 µL of the purified DNA solution to be characterized or 5 µL of one of the 7 dilutions of a of the three positive controls. Then, the 20 µL of each reaction mixture was deposited in one of the wells of a 96-well Hard-Shell® PCR HSP9645 plates, covered immediately with an MicroAmp™ Optical Adhesive Film and the plate was then inserted into a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA) programmed as follows: 15 min of stabilization at 37°C, 2 min at 95°C for the activation of the polymerase enzyme, followed by 50 cycles with 10 s at 95°C for denaturation of all double stranded DNA then 60 s at 60°C for hybridization of the primers and TaqMan probe to gene of interest and elongation of the target DNA by DNA polymerase. The Real-Time PCR lasted a total of 2 h. PCR data was recorded and processed by Bio-Rad CFX Maestro software on Windows.

II.9. Intestinal villi isolation

The intestinal villi were obtained following the protocol of [Guerrero et al. \(2010\)](#) with modifications. Animal experiments were approved by the Ethics Committee of the School of Veterinary Medicine, National University of Colombia, according to the established guidelines. Adult male and female ICR mice (older than 6 months of age), obtained from Faculty of Veterinary Medicine, National University of Colombia, (*Bogotá*, Colombia), were killed by cervical dislocation; their small intestines were removed immediately for enterocyte and intestinal villi preparations. Whole small intestine isolated from an adult mouse was placed in pre-cooled Eagle's minimum essential medium (MEM) (Sigma, St. Louis, MO, USA); the intestinal lumen was flushed using a syringe with 5 mL MEM containing antibiotic/antimycotic solution [final concentration 100 µg.mL⁻¹ kanamycin (Sigma, St. Louis, MO, USA), 100 µg.mL⁻¹ ampicillin (Gibco, Grand Island, NY, USA) and 2.5 µg.mL⁻¹ amphotericin B (Gibco, Grand Island, NY, USA)]. The small intestine was opened longitudinally and cut into 0.5-cm-long segments, which were placed in Petri dishes with 10 mL MEM containing 1.5 mM EDTA, and antibiotic/antimycotic solution. Intestine

fragments were collected in a 15-mL centrifuge tube and incubated for 15 min at 37°C using a constant 200-rpm rate of agitation. Villi/tissue suspension was dispersed by gentle pipetting using a 1-mL micropipette tip with its leading end having been cut off to widen the tip end inside diameter to 2 mm. The dispersed suspension was passed through a sterile metal net (1 mm² pore size), and the filtered cells and tissue were collected in a Petri dish. The material retained in the net was suspended in MEM containing 1.5 mM EDTA and antibiotic/antimycotic solution to be submitted to an additional step of dispersion and filtration. Both filtered preparations were pooled and collected by centrifugation at 600 g for 5 min at 4°C. After the supernatant had been removed, the precipitated material was suspended in 5 mL MEM containing antibiotic/antimycotic solution and washed three times with the same medium. The villi-enriched preparation in MEM containing antibiotic/antimycotic solution was kept at 4°C until assayed for HuNoV infection, for no longer than 1 h.

II.10. *In vitro* cellular infection with HuNoV

Some authors suggest that the VP1 capsid protein needs to be cut for the virus to properly infect cell lines (Ma et al., 2018; Tan and Jiang, 2006). Attempting to cultivate the wild HuNoV, several techniques were tested. One technique was of the intestinal villi-enriched preparation that was plated in 96-well cell culture plates (Nunc) with 100 µL of MEM without fetal bovine serum (FBS). The inoculated villi were placed making sure that comparable amounts of villi were applied to each well. Then, the infection was standardized with NVGII isolated from a stool sample that was negative for NVGI, RV-A, AstV, *Shigella* spp., *Salmonella* spp. and *Cryptosporidium* spp. For this, the sample was filtered through a 0.2 µm cellulose nitrate membrane and it was determined the ideal trypsin concentration to activate the virus. The procedure was performed using different concentrations of trypsin (0 µg.mL⁻¹, 1 µg.mL⁻¹, 5 µg.mL⁻¹, 10 µg.mL⁻¹, 100 µg.mL⁻¹ and 200 µg.mL⁻¹ in PBS) that was added to the sample with HuNoV and it was incubated at 37°C for 20 min. These concentrations were used considering reports for HuNoV (Hardy et al., 1995). Then, trypsin inhibitor was added in the same concentration that trypsin. The dilutions of HuNoV were (1:25, 1:50, 1:100, 1:200 and 1:400) that was added to 100 µL of MEM, in 96-well plates (Nunc) containing the isolated intestinal villi. The cells were cultured in an incubator at 37°C at 5% CO₂ for 12 h, were harvested and fixed in suspension with methanol–acetic acid (3:1 v/v) for 20 min at 0°C, and the infection was evaluated by immunocytochemistry or immunofluorescence, using the anti-norovirus monoclonal antibody (Anti-norovirus MAb).

To confirm that intestinal villus-enriched preparation is adequate to infect and isolate wild HuNoV, the intestinal villus-enriched preparation was plated in 96-well cell culture plates (Nunc) with 100 μL of MEM without fetal bovine serum (FBS) and then inoculated by adding 10 μL of suspension with HuNoV isolated and trypsin-activated ($10 \mu\text{g}\cdot\text{mL}^{-1}$ of trypsin). The HuNoV was recovered from each of the 36 environmental samples that were positive for immunodot-blot and/or RT-qPCR from different sources were tested: air, river water, irrigation water, storm water combined with wastewater, drinking water, leached and chard-leaves. The villi were cultured in an incubator at 37°C at 5% CO_2 , until complete cell lysis was observed, in approximately 12-24 h. To evaluate whether HuNoV was infecting and replicating in the villi, this lysate was frozen and thawed twice, then 10 μL of the lysate was taken, activated with trypsin ($10 \mu\text{g}\cdot\text{mL}^{-1}$) and applied to 100 μL of a new villus culture with MEM, without fetal bovine serum (FBS). To evaluate HuNoV infecting in the villi, it was analyzed by the immunocytochemistry or immunofluorescence 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.

To deduce the replicative cycle of HuNoV, the intestinal villus-enriched preparation with 100 μL of MEM without fetal bovine serum (FBS) was plated in 96-well cell culture plates (Nunc) and was infected with NVGII of the stool sample N° 1 (in [Table 1](#)), dilution 1:50, previously activated with trypsin ($10 \mu\text{g}\cdot\text{mL}^{-1}$ or $100 \mu\text{g}\cdot\text{mL}^{-1}$). Infected villi were cultured in a 5% CO_2 incubator at 37°C and harvested at different time's post-infection (2 h, 4 h, 6 h, 8 h, 10 h and 12 h p.i. (hours post-inoculation)). The villi harvested at each designated hour were centrifuged at 1200 g and the supernatant recovered. MEM without FBS in a volume equivalent to the removed supernatant was added to the pellet and lysed by two cycles of freezing and thawing. The cell lysate or the supernatant was activated with trypsin ($10 \mu\text{g}/\text{mL}^{-1}$ and $100 \mu\text{g}\cdot\text{mL}^{-1}$), added to freshly isolate intestinal villi, and harvested at 12 h.p.i. Infectious titers of cell lysates or the supernatant were determined using immunocytochemistry and capture ELISA assay.

In the search for cell lines to replicate wild HuNoV, in order to use as few mice as possible, two tumor lines were tested based on reports that HuNoVs infect monocyte-macrophages and B ([Bhar and Jones 2019](#); [Jones et al., 2015](#); [Wobus et al., 2004](#)). For this reason, it was evaluated whether wild HuNoVs infected the tumor line U937 cells (ATCC CRL-1593.2), derived from human pleural effusion (histiocytic lymphoma monocyte) or REH (human, African, peripheral blood, leukemia, pre-B cell). First, the infection was standardized with HuNoV isolated from stool sample N° 1 (in [Table 1](#)). For this, in a plate of 96 Wells

(Nunc), 5×10^4 U937 or REH cells in 100 μL of RPMI were infected with 10 μL of NVGII suspension activated with trypsin ($10 \mu\text{g}\cdot\text{mL}^{-1}$). The cells were infected with dilutions of wild HuNoV (1:12.5, 1:25, 1:50, 1:100) and incubated for 36 h at 37°C in incubator with 5% CO_2 and the infection was evaluated by immunofluorescence. The isolated mouse villi, which served as a control, were infected at 1:50 and 1:100 dilutions. The same REH and U937 tumor lines were infected with RV-A Wt1-5, adapted to these tumor lines (Guerrero et al., 2016).

II.11. Antisera preparation

Hartley guinea pig were subcutaneously immunized with 1 mL of a Freund's incomplete adjuvant emulsion containing the purified HuNoV by CsCl ($1 \text{ mg}\cdot\text{mL}^{-1}$), previously boiled for 5 min in a water bath, and then mixed with FIS peptide (FISEAAIIHVLHSR) ($0.5 \text{ mg}\cdot\text{mL}^{-1}$) 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 until use. Control pre-immune sera were collected before immunization.

II.12. 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 pig polyclonal antibodies (generated in the *Molecular Virus Biology Laboratory*; 1:250; 1:500, 1:1000, 1:2000, 1:4000, 1:8000 dilution) against HuNoV or $0.2 \mu\text{g}\cdot\text{mL}^{-1}$ anti-VP1 monoclonal antibody (Norwalk Virus (1D9), sc-53557 Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were added to fixed cells. Following incubation for 1 h at 37°C , the cells were washed twice with PBS and reacted with $0.4 \mu\text{g}\cdot\text{mL}^{-1}$ HRP-conjugate goat anti-guinea pig secondary antibody (SC-2443 Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or secondary HRP-conjugated goat anti-mouse antibody (SC 2005, Santa Cruz Biotechnology Inc.). Peroxidase activity was revealed with aminoethylcarbazole substrate (Sigma, St. Louis, MO, USA). Ten representative fields on coverslips were photographed at $40\times$ 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 immunofluorescence analysis, the Villi were fixed and permeabilized with methanol-acetic acid. Blocking was performed by treating cells with 1% BSA in PBS for 1 h at 37°C. Permeabilized Villi were incubated with primary monoclonal antibodies Norwalk Virus (1D9) ($0.2 \mu\text{g.mL}^{-1}$, sc-53557) or guinea pig polyclonal antibodies (1:250; 1:500, 1:1000, 1:2000, 1:4000, 1:8000 dilution) against HuNoV. Villi were washed three times with PBS before being incubated with a fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG secondary antibody ($0.4 \mu\text{g.mL}^{-1}$, SC-2099) for 20 min at 4°C. Coverslips were mounted onto glass slides and observed in light microscope (VanGuard, Nova-Tech International, Inc., Kingwood, TX, USA) and the percentages of immunoreactive cells were determined as mentioned above. To eliminate autofluorescence, 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 as marked with $1 \mu\text{g.mL}^{-1}$ DAPI stain.

U937 or REH cells infected with HuNoV after 36 h p.i., to 37°C in a CO₂ incubator at 5% were harvested and fixed with paraformaldehyde at 4% for 20 min at 20°C. After washing with PBS 3 times, fixed cells were placed onto coverslips and allowed to dry for 1 h at 50°C. Then $0.2 \mu\text{g.mL}^{-1}$ anti-VP1 monoclonal antibody (Norwalk Virus (1D9), sc-53557 Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were added to fixed cells. Following incubation for 1 h at 37°C, the cells were washed three times with PBS and reacted with HRP-conjugate-goat anti-mouse secondary antibody ($0.2 \mu\text{g.mL}^{-1}$ Sc2005, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Peroxidase activity was revealed with aminoethylcarbazole substrate (Sigma, St. Louis, MO, USA). 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 immunofluorescence we perform this same procedure except that secondary antibody was anti-mouse with phycoerythrin. Coverslips were mounted onto glass slides and observed in a light microscope (VanGuard, Nova-Tech International, Inc., Kingwood, TX, USA). The percentages of immunoreactive cells were determined.

II.13. Virus purification by cesium chloride

NV GII from air sample of a house terrace, coming from the fourth passage of infection and villi obtained from three mice, was purified from the intestinal villus-enriched preparation, mixed and collected in a single bottle with 60 mL of culture medium, showing a complete cytopathic effect. The lysate was emulsified by vortexing with one-third volume of

trichlorotrifluoroethane (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 MgCl₂ and 5 mM CaCl₂); the aqueous phase was centrifuged through 1 mL 30% (w=v) sucrose in TBS at 150000 g (Sorvall TST 60.4 rotor) for 1.5 h at 4°C. Viral particles were suspended 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 280000 g for 1.5 h at 4°C. Visible bands were collected and then diluted with TBS before pelleting at 100000 g for 1.5 h at 4°C. Virus pellet was suspended in sterile phosphate buffered saline (PBS).

II.14. ELISA

In order to determine the infectious curve of NVGII in isolated mouse gut villi, the infection was evaluated by indirect ELISA. Each well of a 96-well ELISA plate was coated with cell lysate samples of HuNoV (400 ng.well⁻¹.100 µL⁻¹) 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 the primary antibody and HRP-conjugated goat anti-mouse as the secondary antibody (0.133 µg.mL⁻¹ Sc2005, Santa Cruz biotechnology Inc., Santa Cruz, CA, USA). The reaction was developed using ortho-phenylenediamine (OPD) (1 mg.mL⁻¹) (Pierce) and H₂O₂ (0.04%) in 100 mM sodium citrate buffer, pH 5.0.

Similarly, ninety-six-well ELISA plates (Nunc) were coated with polyclonal guinea pig-antibodies (generated in our lab; 1:1000 dilution) against HuNoV particles for 12 h at 4°C. Coated plates were blocked with 5% skimmed milk in PBS for 1 h at 37°C. The HuNoV-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 X 1000, 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⁻¹.100 µL⁻¹); plates were incubated for 2 h at 37°C and then washed with PBST (PBS containing 0.05% Tween 20, (Sigma, St. Louis, MO, USA) three times before adding 0.2 µg.mL⁻¹ anti-VP1 monoclonal antibody (Norwalk Virus (1D9), sc-53557 Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). After three PBST washes, a bovine anti-mouse HRP-conjugate (sc-2371, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) 0.133 µg.mL⁻¹ in PBST, containing 0.5% skimmed milk, was added to plates and incubated for 1 h at 37°C. Plates were washed three times with PBST and were added OPD (Pierce,

Rockford, IL, USA) peroxidase substrate ($0.4 \text{ mg}\cdot\text{mL}^{-1}$) 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 HuNoV-infected sample readings. The results were presented as the mean of duplicates; all experiments were repeated three times.

II.15. Cell viability test

Villus cell viability was measured using Trypan blue solution (Sigma–Aldrich, St. Louis, MO, USA) in an exclusion assay. The HuNoV-infected villi and non-infected villi were assessed after different p.i. times. Namely, 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 quantified in a Neubauer chamber under a light microscope.

II.16. Western blotting (WB)

Gradient purified HuNoV in cesium chloride was used to analyze viral proteins. To 25 μL of the sample was added trypsin ($10 \text{ }\mu\text{g}/\text{mL}$), incubated for 20 min at 37°C and the second aliquot (25 μL) was not treated with trypsin. Both samples were mixed with Laemmli buffer and run on a 12% SDS-PAGE gel. After WB, PVDF membranes were probed with $0.2 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$ anti-VP1 monoclonal antibody (Norwalk Virus (1D9, sc-53557 Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) followed by treatment with HRP-conjugated bovine anti-mouse (sc-2371, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and AEC substrate. Peroxidase activity was revealed with aminoethylcarbazole substrate.

II.17. Statistical analysis

Results are expressed, as mean \pm 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 $P < 0.05$ considered indicating a significant difference.

III. Results

III.1. HuNoV detection from human stools and environmental samples

The 37 faeces samples (including 34 samples from children <5 years and 3 from elderly >75 years) were initially analyzed using the immunodot-blot technique with monoclonal antibodies (Table 1 and Figure 1). Thirteen of these samples were positive for enteric viruses, of which 8 were RV-A only, 2 HuNoV only and 3 were both viruses. Performing subsequent real-time RT-PCR assays to identify HuNoVs by genome group, we observed that of the 5 samples that had tested positive for HuNoV with immunodot, 2 contained genome copies (GC) of NV GII above the calculated detection threshold (300-1200 GC.g⁻¹ human stool), while the three others did not reach the threshold for both NVGI and NVGII (Table 1). Of the 2 positive samples for NVGII, one did not contain the other viruses tested by immunodot with monoclonal antibodies, i.e. RV-A and AstV. This sample was used to standardize the trypsin concentration to activate HuNoV, and also, to study the replicative cycle in the isolated villi of the mouse intestine.

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 that were positive by immunodot-blot only, by RT-qPCR only

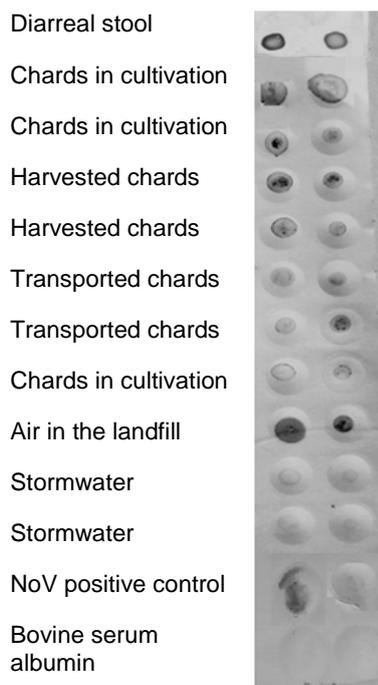


Figure 1: Immunodot-blot detection of HuNoV onto polyvinylidene fluoride (PVDF) membrane, after incubation for 1 h at 37°C of 20 µL of partially purified viral suspensions supplemented with anti-VP1 monoclonal antibody, and revealed by adding *in fine* secondary HRP-conjugated goat anti-mouse antibody.

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 (+)/(-)	NVGII*	RV-A	<i>Shigella</i> spp.	<i>Cryptosporidium</i> 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	

Table 1: Pathogens detection from human faeces samples using monoclonal and polyclonal antibodies and real time PCR. The pathogen concentrations on faeces samples (GC.g⁻¹) are given when they are above the detection threshold (about 300-1200 GC.g⁻¹ human faeces).

*: NVGI was never detected in the stools.

**:: sample used for the standardization of trypsin concentration for HuNoV activation and for the study of NVGII replicative cycle.

and by both methods (immunodot-blot and RT-qPCR), respectively (**Table 2**). Of the 14 HuNoV-positive by RT-qPCR, 6, 6 and 2 samples were positive for NVG1 only, NVG2 only, and both NVG1 and NVG2, respectively. 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.

Source	Low rainfall months*			Medium rainfall months*			High rainfall months*			
	no HuNoV	NVGI	NVGII	NVGI & NVGII	no HuNoV	NVGI	NVGII	no HuNoV	NVGI	NVGII
----- RT-qPCR (Immunodot-blot)-----										
Tunjuelito river water	<u>1+2</u> (1+2)		1 (0)	1 (0)	5 (5)					
Stormwater	<u>+1</u> (+1)		2 (0)	1 (0)	<u>3+1</u> (3+1)		1 (0)			1 (0)
Irrigation waters		1 (1)						1 (1)		
Tap water	1 (1)									
Leachate treated in wastewater plant	2 (2)									
Chards during cultivation in farms		1 (0)				1 (1)		1 (1)		
Harvested chard leaves		1 (1)			1 (1)					
Transported chard leaves in truck	1 (1)					1 (1)				
Chard leaves from wholesale market								1 (1)		
Air from Doña Juana landfill		1 (0)						1 (1)		
Air from leachate treatment plant	1 (1)									
Air from housing terraces	2 (2)		1 (0) **					1 (1)		

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

In the area where this research was carried out, there is a sanitary landfill, where the

waste from the city of *Bogotá*, Colombia, is dumped, called "*Doña Juana*". For this reason, the presence of the HuNoV of the different environmental matrices (water, vegetables and air) was evaluated by immuno-dot blot with respect to the distance to the sanitary landfill. 63% of the samples (26/41) were positive within a 3-km radius of the landfill (air of the landfill, of housing terraces and leachate treatment plant; waters from *Tunjuelito* river and storm water combined with wastewater; leachate treated in wastewater plant; a school drinking water; and chards grown near to the landfill), while 38% (5/13) were positive in sources located beyond (*Corabastos* wholesale market, irrigation water, chard leaves at cultivation and harvest, and chard leaves transported in truck).

III.2. Culture and norovirus infection in isolated villi

The culture of non-infected isolated villi with the protocol retained in our study seems reliable as about 50% of cells excluded trypan blue at 24 h p.i. (Figure 2); for information, the fraction of viable cells seemed to be affected by HuNoV infection (Figure 2).

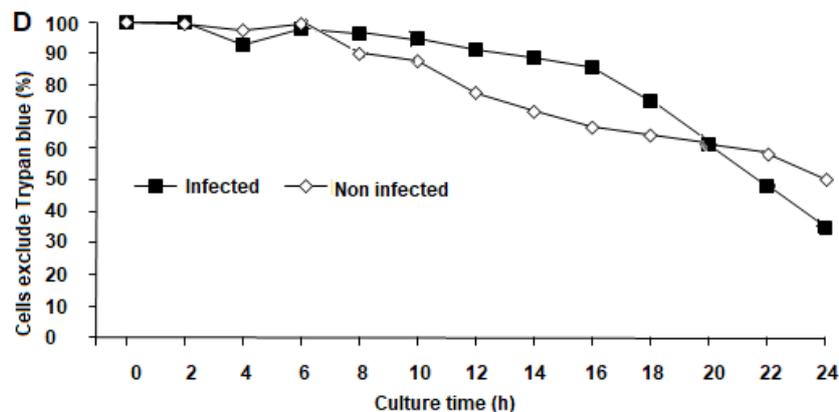


Figure 2: Villus cell viability measured using Trypan blue solution as a function of time for villus cells cultivated with and without HuNoV infection in an exclusion assay in a Neubauer chamber under a light microscope.

The model of isolated mouse villi was infected with HuNoV isolated from human faeces and analyzed by the immunofluorescence technique (Figure 3). Different concentrations of trypsin were used to determine the ideal concentration to activate HuNoV. It was found that from $10 \mu\text{g.mL}^{-1}$ there was no difference in the infective capacity (Figure 4).

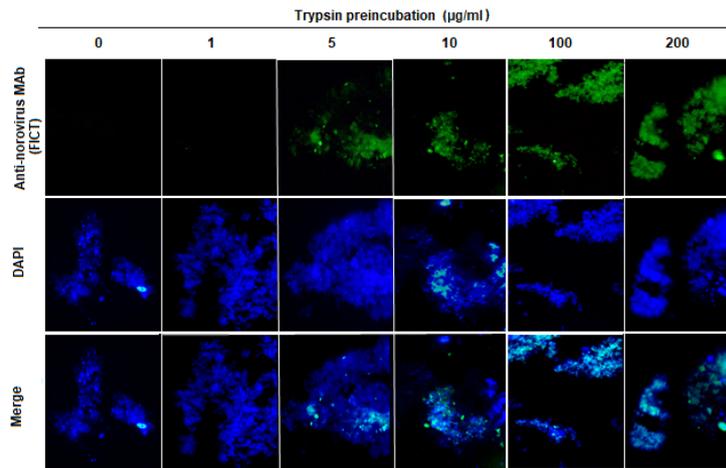


Figure 3: Observation under a light microscope with 40× magnification of immunofluorescence assay (accumulating HuNoV structural antigen) in isolated intestinal villi from intestinal sections (duodenum, jejunum, ileum) that were seeded into 96-well culture plates, inoculated with trypsin-activated HuNoV at a dilution 1:25, and harvested 12 h p.i..

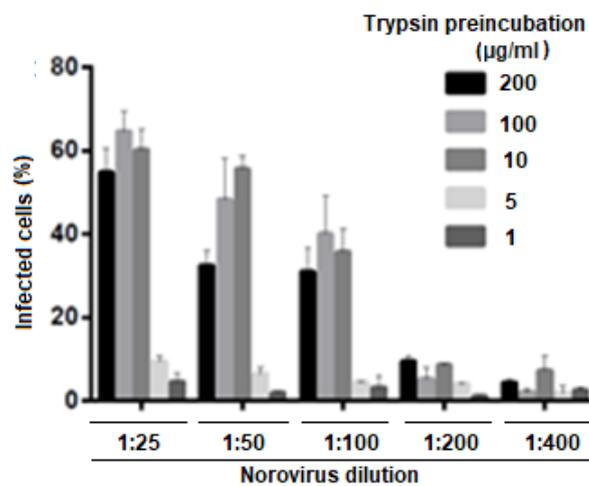


Figure 4: Percentage of villus cells positive to HuNoV from immunocytochemistry, as functions of HuNoV and trypsin concentrations in isolated intestinal villi from intestinal sections (duodenum, jejunum, ileum) that were seeded into 96-well culture plates, inoculated with trypsin-activated HuNoV, and harvested at 12 h p.i.. Observations were performed under a light microscope with 40× magnification. Mock-inoculated villi were used as control. Error bars represent the standard error of the means from triplicate samples (n= 3 wells).

Subsequently, to confirm that the isolated mouse gut villus model is suitable for infecting and replicating HuNoV, the villi were infected with 10 μ L of HuNoV isolated and concentrated from each of the environmental sources, as described in materials and methods. The infection was evaluated by immunocytochemistry and immunofluorescence techniques, using monoclonal antibody. All isolated viruses were found to adequately infect villi, with an infection rate of 75%-90% when tested by immunocytochemistry (Figures 5a-b). Similar results were obtained when tested by immunofluorescence, with a percentage of villus infection exceeding 90% for HuNoV from all sources examined (Figure 6a-b).

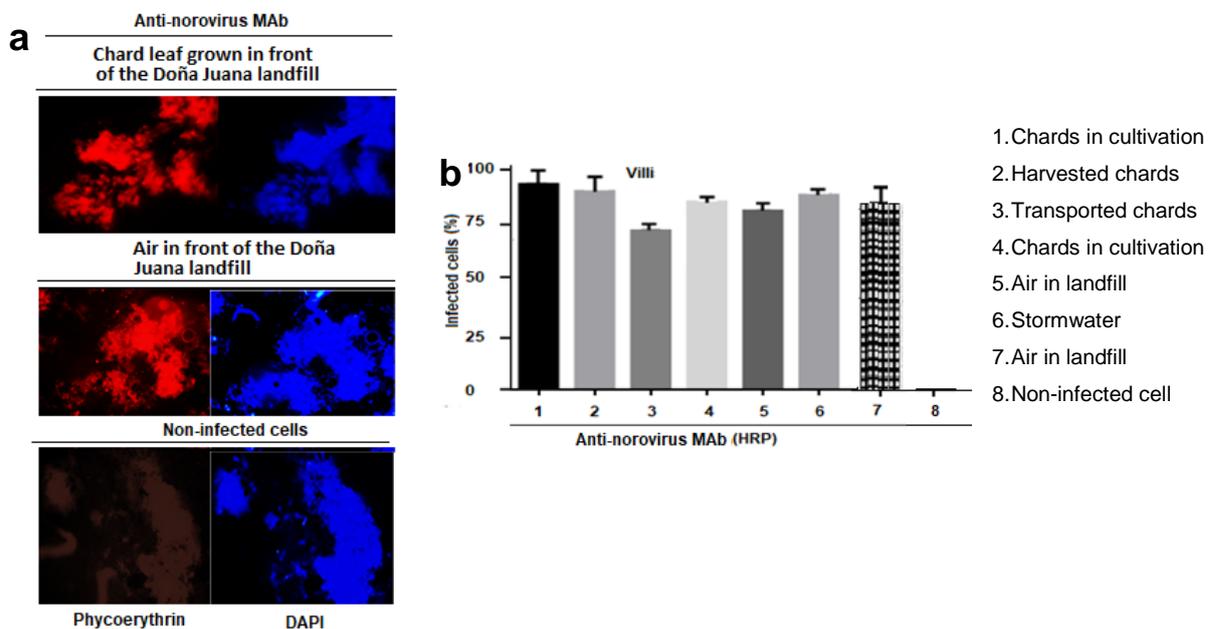


Figure 5: Accumulating HuNoV structural antigen in isolated intestinal villi from intestinal sections seeded into 96-well culture plates, inoculated with trypsin-activated HuNoV from samples of different environmental matrices (water, plants and air), harvested at 12 h.p.i., and examined immunocytochemically for the percentage of villus cells positive to HuNoV structural antigens. (a) microscopic observations under a light microscop with 40 \times magnification after villi harvest; (b) percentage of villus cells positive to HuNoV structural antigens estimated after 1 h additional incubation at 37 $^{\circ}$ C with anti-VP1 monoclonal antibody, and secondary HRP-conjugated goat anti-mouse antibody SC2005. Mock-inoculated villi were used as control. Error bars represent the standard error of the means from triplicate samples (n= 3 wells).

a

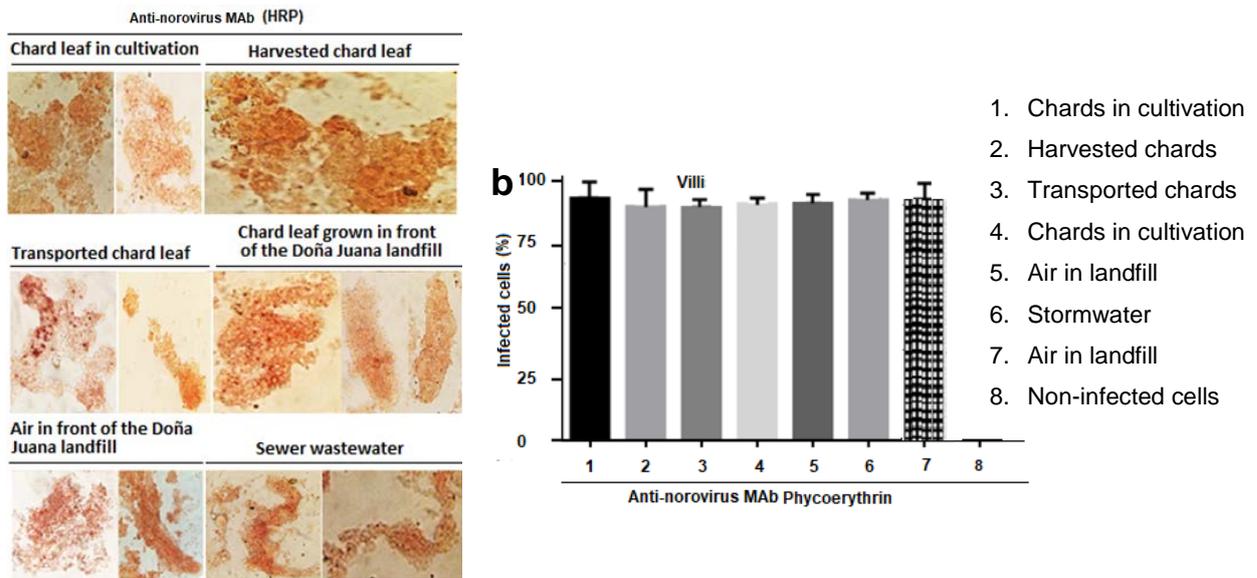


Figure 6: Accumulating HuNoV structural antigen in isolated intestinal villi from intestinal sections seeded into 96-well culture plates, inoculated with trypsin-activated HuNoV from samples of different environmental matrices (water, plants and air), harvested at 12 h.p.i., and examined by immunofluorescence for the percentage of villus cells positive to HuNoV structural antigens. (a) microscopic observations under light microscop in 40× magnification; (b) percentage of villus cells positive to HuNoV structural antigens estimated after 1 h additional incubation at 37°C with anti-VP1 monoclonal antibody, and secondary HRP-conjugated goat anti-mouse antibody with phycoerythrin. Mock-inoculated villi were used as control. Error bars represent the standard error of the means from triplicate samples (n= 3 wells).

Two tumor cell lines (REH and U937) were also tested for replication of wild HuNoV. For this purpose, 50000 cells from the tumor lines in 100 μL were infected with HuNoV isolated from feces (sample 1, **Table 1**), in dilutions 1:12.5, 1:25, 1:50, 1:100. The isolated mouse villi that served as controls were infected at 1:50 and 1:100 dilutions. The infection percentage was very low, with dilution 1:12.5 the infection was around 5% for the U937 line and 3%-10% for REH (**Figure 7a-b**, **Figure 8**). As a positive control of the infection, the same tumor cell lines were infected with RV-A Wt1-5 in a multiplicity of infections (MOI 0.8), adapted to those tumor lines (**Guerrero et al., 2016**) presenting an infection of 70% for REH and 75% for U937. Another control used was to infect the isolated villi with the 1:50 dilution, which presented an infection of around 60%.

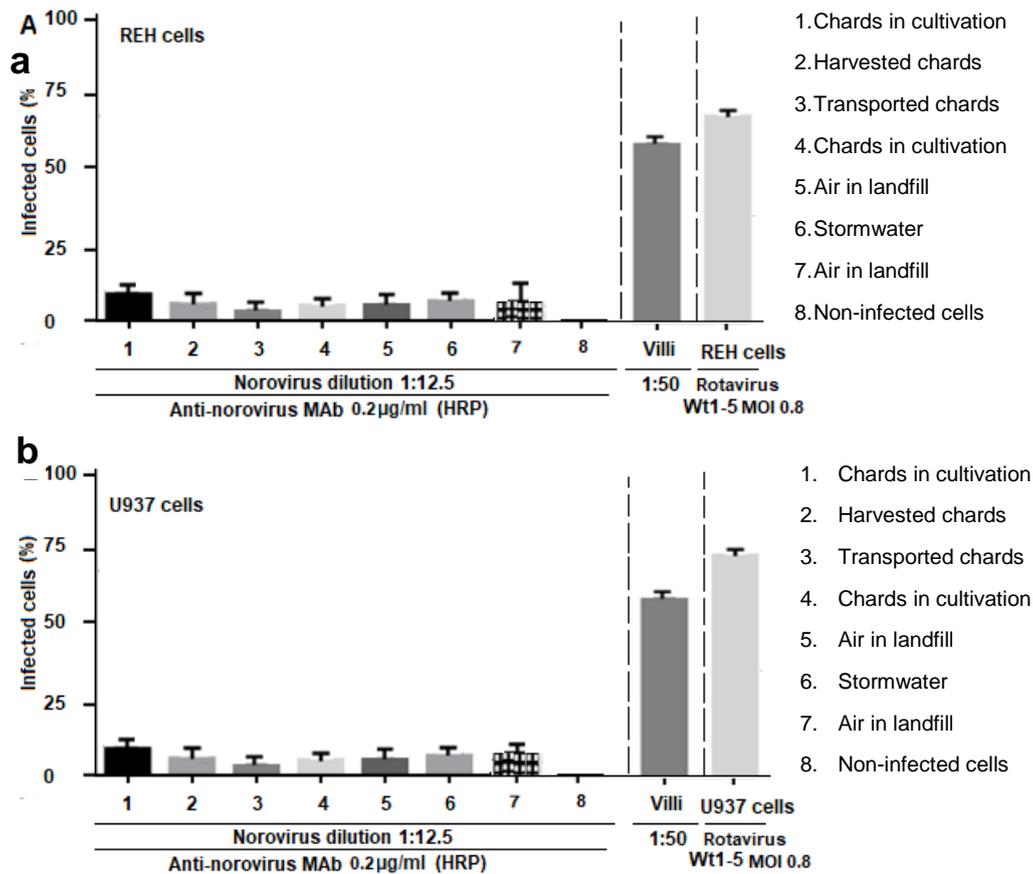


Figure 7a-b: Accumulating HuNoV structural antigen in REH and U937 cells. REH or U937 were seeded into 96-well culture plates, inoculated with trypsin-activated HuNoV, harvested at 26 h.p.i., and examined immunocytochemically for the percentage of cells positive to HuNoV structural antigens. For this last examination, cells were incubated for 1 h at 37°C with anti-VP1 monoclonal antibody, with thereafter the addition of secondary HRP-conjugated goat anti-mouse antibody. REH and U937 cells were inoculated with rotavirus as control.

III.3. HuNoV growth curve in isolated intestinal villi

In order to determine the infectious curve of NVGII (from sample 1, [Table 1](#)) in the isolated villi of mouse intestine, these were infected with a 1:50 dilution and harvested every 2 h. That is, NVGII was added to the cells and they were harvested at 0, 2, 4, 6, 8, 10 and 12 h p.i.. The aliquots were frozen and thawed twice and the lysate harvested every 2 h was added to new villi. All aliquots were seeded into the new villi simultaneously and harvested after 12 h p.i.. The infection was evaluated by immunocytochemistry and by indirect ELISA, using

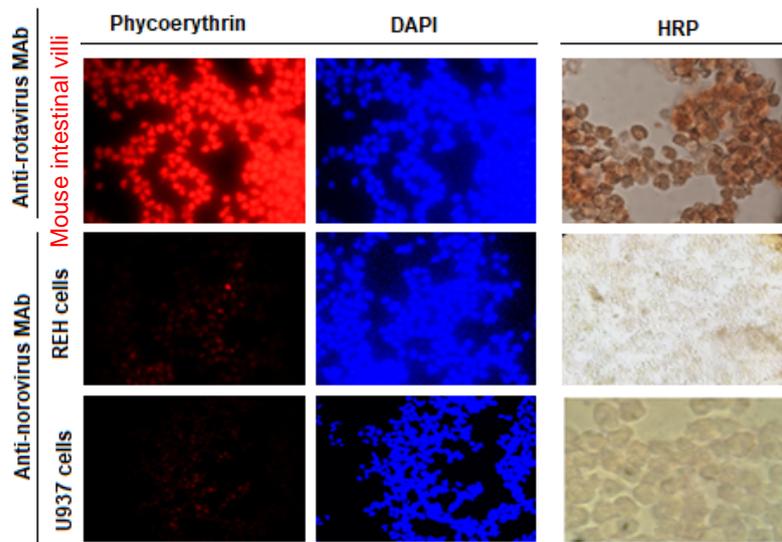


Figure 8: Accumulating HuNoV structural antigen in REH and U937 cells seeded into 96-well culture plates, inoculated with trypsin-activated HuNoV, harvested at 26 h.p.i., and examined by immunofluorescence for the percentage of cells positive to HuNoV structural antigens. Villi cells were inoculated with RV-A Wt1-5 as control.

monoclonal antibody in both techniques. By immunocytochemistry a rising peak is observed up to 8 h p.i., and it is stabilized up to 12 h p.i. (**Figure 9a**). By indirect ELISA, the rising peak is observed up to 10 h p.i., both in the supernatant and in the cell pellet lysate (**Figure 9b**). The aliquots were frozen and thawed twice and the lysate harvested every 2 h was added to new villi. All aliquots were seeded into the new villi simultaneously and harvested after 12 h p.i.. These results suggest that the 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 leave the enterocyte.

To determine that HuNoV grown repeatedly in the mouse isolated villus model maintains the characteristics already established by electrophoresis and western blot, it was analyzed by these techniques after treating human NVGII (from sample 1, **Table 1**) with $10 \mu\text{g}\cdot\text{mL}^{-1}$ trypsin. It was found to have two characteristic bands of 58 and 38 kDa after trypsin treatment (**Figure 10**).

III.4. Isolated villi from mouse intestine allow a good amount of wild HuNoV to be obtained for purification by CsCl

We sought to have enough virus to purify by CsCl gradient and to carry out biochemical studies or to generate polyclonal antibodies. For this purpose, NVGII isolated and

concentrated from the air on the terrace of a house located to 3 km from the *Doña Juana* landfill (**Table 2**) was used to infect isolated intestinal villi from mouse. The NVGII 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 was achieved in approximately 36 hours. The lysates were emulsified with trichlorotrifluoroethane and purified by means of CsCl. The NVGII recovered was used to generate polyclonal antibodies in guinea pig. The antibodies generated recognize HuNoV isolated from different environmental sources and were titrated in immunocytochemistry techniques with 1:4000 dilutions (**Figure 11**) and capture ELISA (**Figure 12**), with 1:1000 dilutions. Villi infected with HuNoV were harvested and fixed in suspension with methanol–acetic acid (3:1 v/v).

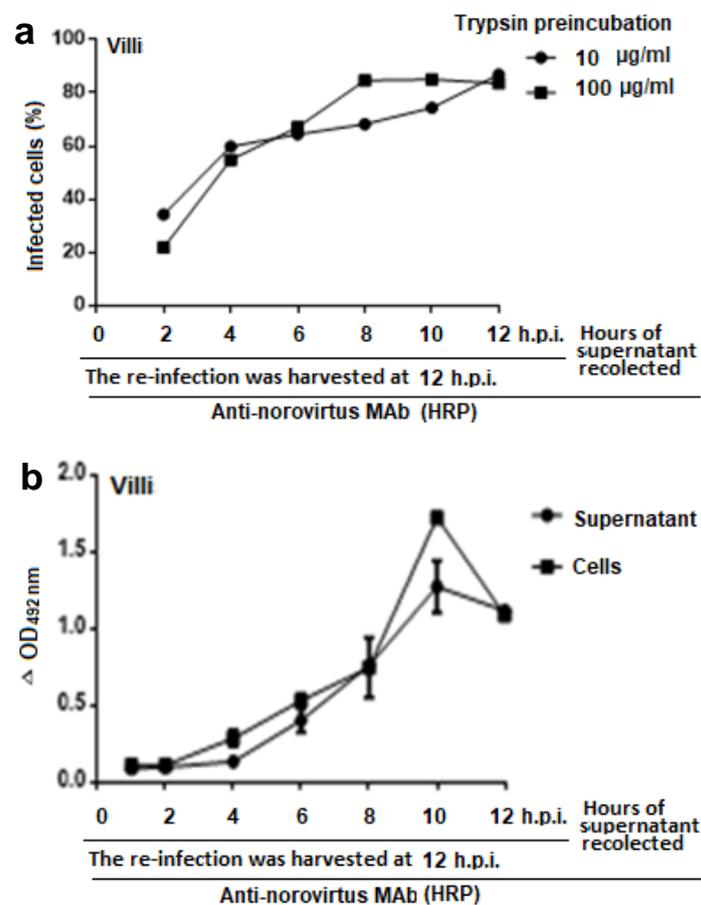


Figure 9a-b: Growth curve of NVGII in isolated intestinal villi placed into 96-well culture plates and then inoculated with trypsin activated NVGII. Mock-inoculated villi were used as control. Error bars represent the standard error of the means from triplicate samples (n= 3 wells). The percentage of villus cells positive to NVGII structural antigens on collected villi were examined (a) immunocytochemically and (b) by ELISA.

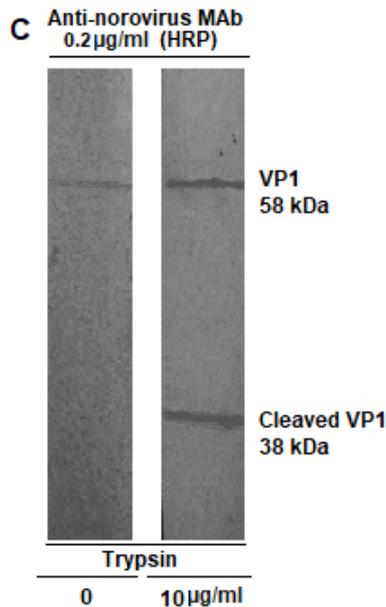


Figure 10: Western Blotting. PVDF membranes were probed with anti-VP1 monoclonal antibody followed by treatment with HRP-conjugated bovine anti-mouse peroxidase activity was revealed with aminoethylcarbazole substrate.

Fixed cells were placed onto coverslips and allowed to dry for 1 h at 50°C. Guinea polyclonal antibodies against HuNoV or anti-VP1 monoclonal antibody were added to fixed cells. The cells were reacted with HRP-conjugate goat anti-guinea pig secondary antibody or secondary HRP-conjugated goat anti-mouse antibody.

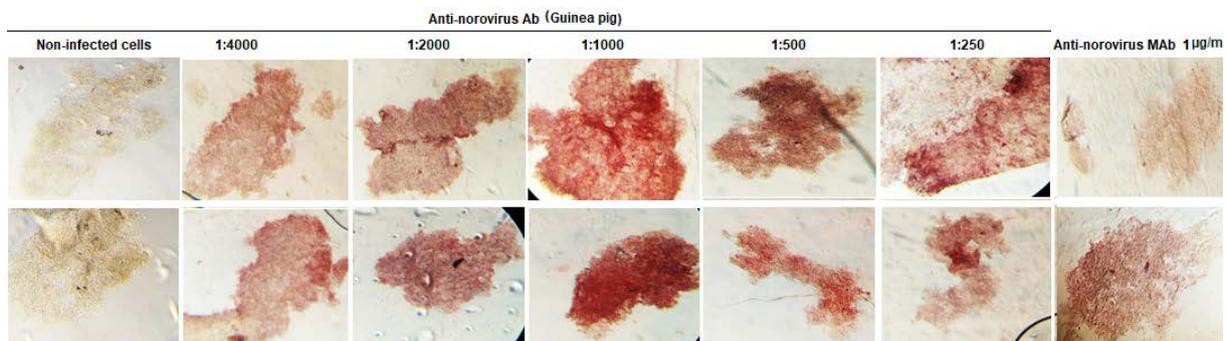


Figure 11: Peroxidase activity revealed with aminoethylcarbazole substrate, in villi infected with HuNoV harvested and fixed in suspension with methanol-acetic acid (3:1 v/v), after having placed the fixed cells onto coverslips and allowed them to dry for 1 h at 50°C. Guinea pig polyclonal antibodies against NVGII or anti-VP1 monoclonal antibody were added to fixed cells. The cells were reacted with HRP-conjugate goat anti-guinea pig secondary antibody or secondary HRP-conjugated goat anti-mouse antibody. Representative fields were photographed at 40× magnification.

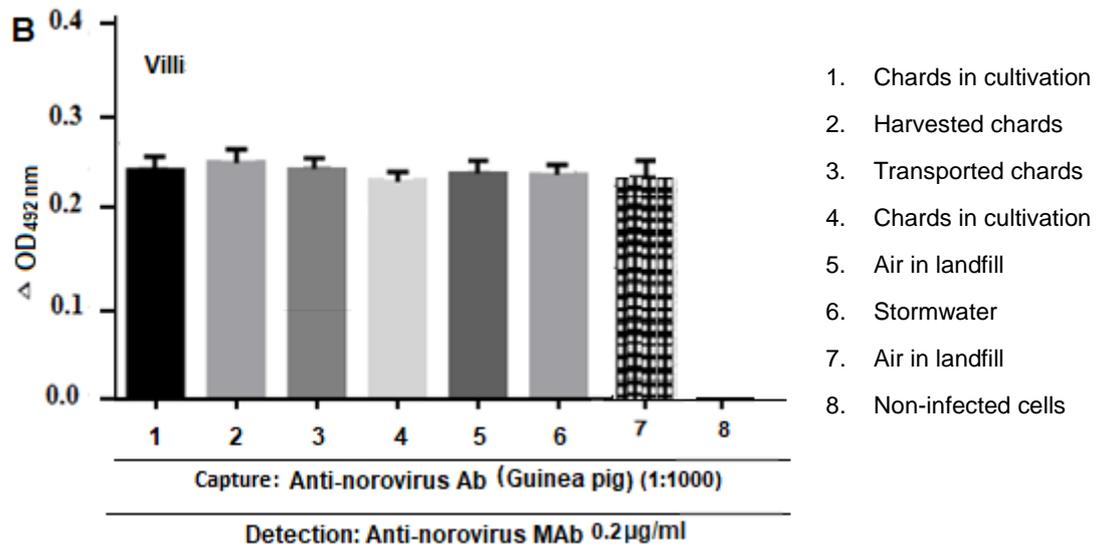


Figure12: ELISA plates were coated with polyclonal guinea pig-antibodies against NVGII particles for 12 h at 4°C. Villus lysates were added to wells and were incubated for 2 h at 37°C and then adding 0.2 μg.mL⁻¹ anti-VP1 monoclonal antibody. bovine anti-mouse HRP-conjugate was added to plates and incubated for 1 h at 37°C. Plates were added OPD (Pierce, Rockford, IL, USA) peroxidase substrate. Absorbance was read at 492 nm after 20 min. Absorbance values corresponding to lysed without virus antigen were subtracted from all HuNoV-infected sample reading. The results were presented as the mean of duplicates; all experiments were repeated three times.

IV. Discussion and Conclusion

HuNoV is a highly contagious virus and the study of this virus has been hampered by the lack of *in vitro* culture methods or animal models, even though its discovery was in 1972 by immunoelectronic 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 type O blood (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 comes closest to *in vitro* cell culture, which mimics *in vivo* conditions, is isolated villi of mouse small-bowel. So far, there are no report of a model that allows successful replication of wild HuNoV collected from diverse sources such as air, water, or plants. None of the cell culture combinations reported so far have been successful *in vitro* in replicating of wild HuNoV. For this reason, attempts to detect and isolate environmental viruses have been limited to biochemical detection, using techniques such as immunoassays or detecting the genome by RT-PCR, but without determining whether the detection is biologically active or infective (Rodríguez et al., 2009; Duizer et al., 2004; Karim and LeChevallier, 2004). Using isolated villi from mouse small intestine, we found that 36 samples from environmental sources and 5 from human faeces were positive. Of these, 22 samples from environmental sources were positive for HuNoV by immunodot-blot technique only, 10 by RT-qPCR technique only, and 4 by both techniques. On the other hand, the 5 human faeces samples were all positive for the presence of HuNoV by immuno-dot technique, of which 2 were subsequently confirmed for NVGII by RT-qPCR technique. The most relevant thing to highlight is that most samples detected as positive, by immunodot or RT-qPCR, presented biologically active virions (41/45). This means that the isolated villus model of the mouse small intestine is 100% effective and efficient to be infected and to generate wild virions of human origin. This suggests that the receptors and basic cellular biochemistry necessary for efficient entry and replication of human wild norovirus are found in the villi cells of the mouse. The various combinations of cell cultures reported so far have probably not been as successful in producing *in vitro* norovirus replication, because it is difficult to determine which steps are critical for norovirus replication. Specific characteristics of the host cells, the virus, or both are probably required. Perhaps, for this reason, we were not successful in trying to replicate norovirus in tumor lines such as U937 or REH. However, they recently reported the successful cultivation of multiple strains of HuNoV in enterocytes, in cultures of unprocessed human intestinal monolayers, derived from stem cells (Ettayebi et al., 2016). In this model, bile was a critical factor needed for strain-dependent human norovirus replication. The authors are of the opinion that the lack of adequate expression of blood group antigen ("histoblood") in intestinal cells restricts virus replication. In our model, it was not necessary to use any reagents, except to activate the virus with trypsin, for the infectious success. We only observed that some isolates took longer to lysate the cells (12-24 h), probably because of the reduced number of virions present in the isolate, but all the viral isolates, from the different sources analyzed, infected the intestinal villi of mouse. A disadvantage of this model of intestinal villi isolated from mouse is that the number of cells in each villi cannot be

accurately quantified. When the cells of the villi are broken down, a process of apoptosis begins in an average of approximately 2 hours, in what is denominated anoikis. This drawback makes it difficult to calculate the number of particles generated or the multiplicity of infection (MOI).

In conclusion, the model of intestinal villi isolated from mouse is effective for the study of human wild HuNoV infection and replication from faeces and environmental matrices. Treatment with 10 $\mu\text{g.mL}^{-1}$ trypsin is required to promote HuNoV infection in isolated mouse villi. In this model the human wild norovirus presents an average replicative cycle of 10 h although viral particles with infectious capacity are found from 2h.p.i., and the virions leave of 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, although it has the disadvantage of not being able to calculate the number of particles generated or the multiplicity of infection (MOI). In this model the human wild norovirus presents an average replicative cycle of 10h although viral particles with infectious capacity are found from 2 h p.i..

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Chapter V:

Risks of primary and secondary infections in households in *Usme (Bogotá)*; impact of lifestyle

Once completed, the work presented in this chapter may lead, in reduced form, to the submission of the article:

Jordan-Lozano J.S., Renault P. 2020. Risks of primary and secondary infections in homes in Usme (Bogotá); impact of lifestyle.
to an internationally peer-reviewed journal or to a Colombian scientific journal.

Abstract

Acute gastroenteritis (AGE) are responsible for substantial morbidity, mortality and healthcare costs, which vary with their aetiology, as well as age and health status of infected persons, individual practices (food, hygiene and health), and the socioeconomic context, including the access to care. The objective of this work was to enrich the characterization of human living contexts and individual food, hygiene and health practices to better circumvent the transmission of AGE contaminations in some neighbourhoods of *Usme* district in *Bogotá* (Colombia) which are located less than 2 km northeast of the *Doña Juana* landfill. Surveys were carried out in 133 households. Seventy-five questions successively addressed household inhabitants, socioeconomic conditions, food practices, hygiene practices, AGE cases and health practices to treat these cases. They were analysed by various tools, including principal component analysis and modelling. Surveys have shown that the annual frequency of AGE decreases with increasing age; AGE seemed less common in households with elderly people, possibly due to stricter food hygiene practices. A transmission model of acute gastroenteritis distinguishing contamination from outside the households and contaminations between people in the same households did not show significant differences between neighbourhoods. Used to simulate numerical experiments, it suggests working on much higher numbers of surveys.

I. Introduction

Acute gastroenteritis (AGE) affects a significant proportion of the global population each year: about 33% of the French population ([Van Cauteren et al., 2012](#)), 55% of the US population ([Lively et al., 2018](#)), and probably more in Colombia, Brazil, Peru and Mexico in view of the data of [Troeger et al. \(2017\)](#) concerning only acute diarrhea, although data for France and the US are surprisingly low in this last work. AGE is responsible for substantial morbidity, mortality and healthcare costs ([Troeger et al., 2018](#); [Bartsch et al., 2016](#); [Hoffman et al., 2015](#)), which vary with its aetiology ([Hoffman et al., 2015](#)), as well as the age, health status and access to care of infected persons ([Atchison et al., 2016](#); [Ruiz-Rodríguez et al., 2012](#)). Hence the interest of studies aimed at reducing AGE importance.

The proportions of direct transmissions (from person to person) and indirect transmissions *via* water, food, air and inert surfaces depend on the pathogens ([WHO, 2015](#)) according to their ability to cause a zoonosis ([Chlebicz and Śliżewska, 2018](#); [Pumipuntu and](#)

Piratae, 2018) and survive outside their host(s) (Van Elsas et al., 2011), and on human individual food, hygiene and health practices (Lee et al., 2017). While most viruses (e.g. noroviruses and rotaviruses) causing AGE are thought to infect humans only (Bányai et al., 2018), several other human AGE pathogens also infect animals, such as *salmonella* spp. that infect birds (Chlebicz and Ślizewska, 2018), *cryptosporidium* spp. that infect cattle (Pumipuntu and Piratae, 2018) and *shigella* spp. that infect not only people and non-human primates (McLennan et al., 2018) but also fresh vegetables (Jo et al., 2019). Direct and indirect transmissions of human AGE pathogens are affected by individual practices (Lane et al., 2019) relating to hygiene (personal hygiene, cleanliness of living spaces) (Presterl et al., 2019), food (foodstuffs, purchasing places, handling and preparation) (Pérez-Rodríguez et al., 2019; Mitakakis et al., 2004), and health in response to AGE cases (Van Cauteren et al., 2012). These practices and the resulting microbial risks depend on the climate, economy, culture and regulations (Atkins and Bowler, 2016). Regional variations of such contexts explain regional variations in the relative importance of human AGE pathogen transmission routes. For the ‘AMR B’ geographical area which includes most Latin American countries, direct transmissions would account for about 72%, 15%, 51%, and 35% of the transmissions of norovirus, non-typhoid *salmonella enterica*, *shigella* spp. and *cryptosporidium* spp., respectively (WHO, 2015).

Some specificities of Colombia must be taken into account. First, soiled toilet papers and soiled diapers for babies and adults are disposed in landfills, without being systematically covered immediately afterwards with clay or soil, leading to potential risks of enteric pathogen aerosolization (Chapter IV; Girardin et al., 2016). Second, the intake of vegetables and fruits in Colombia ($35 \text{ kg.p}^{-1}.\text{y}^{-1}$), which is well below that in neighbouring countries like Venezuela ($84 \text{ kg.p}^{-1}.\text{y}^{-1}$) and Mexico ($73 \text{ kg.p}^{-1}.\text{y}^{-1}$) (Guarín, 2013), is largely restricted to a handful of relatively cheap products (Guarín, 2013) that may be irrigated by surface freshwaters heavily contaminated with faecal indicators (Heliodoro and Bustos-López, 2018) and human enteric pathogens (FAO, 2017) due to the discharge of untreated or inadequately treated wastewater into rivers or marshes (Chapter IV). Third, inhabitants belonging to low-income and middle-income strata face significant barriers to accessing health care, even with medical expense subsidies (Alvarez et al., 2011), resulting from inequalities among people aggravated by the great reform of the Colombian health care system carried out in 1993 (Camargo Plazas, 2018), from the lack of awareness about the Colombian healthcare system and their rights to health care (Ayala-García, 2014; Delgado Gallego and Vázquez-Navarrete, 2013), and from difficulties to access to health services in terms of procedures to obtain a

medical appointment, distance from health centers and lack of money (Ayala-García, 2014). Many people prefer going to emergency departments for conditions they consider treatable in a primary care setting due to their worse perceptions of health system performance and quality (Macinko et al., 2016; Ayala-García, 2014). Upstream factors affect these practices and the resulting risks: in particular Law 100 of 1993 on health insurance (Ayala-García, 2014), Decree 1594 of 1984 (*Ministerio de Agricultura*) on the use of water (including for agriculture) (Heliodoro and Bustos-López, 2018), Decree 1575 of 2007 (*Ministerio de Salud* and *Ministerio de Ambiente*) on the quality of water intended for human consumption, and Decree 3075 of 1997 (*Ministerio de Salud*) on the quality of food.

Efforts to take into account societal and individual practices in Quantitative Microbial Risk Assessment (QMRA) (Membre and Guillou, 2016) remain sketchy (Chandrasekaran and Jiang, 2018). Most practices and their determinants are ignored, e.g. the fruit and vegetable consumption affected by education, local beliefs and the cultural organization of meals (Kremer-Sadlik et al., 2015; Kovacs and Ravachol, 2019), the use of water affected by the perception of its quality (Busca and Lewis, 2019), and physician consultation affected by Public Health policy (Van Cauteren et al., 2012; Tam et al., 2012). Thus, one of the limits to the modelling of AGE transmissions lies in the lack of knowledge about people lifestyle.

The objective of this work was therefore to enrich the characterization of human living contexts and individual food, hygiene and health practices to better circumvent the transmission of AGE contaminations. It focused on some neighbourhoods of *Usme* district in *Bogotá* (Colombia) which are located less than 2 km northeast of the *Doña Juana* landfill.

II. Materials and Methods

II.1. Survey zone

The study focused on some neighbourhoods of *Usme* district in *Bogotá*, the capital of Colombia. *Bogotá* is at about 2640 m above sea level and has a subtropical highland climate (Cfb in the Köppen-Geiger climate classification) (Chapter IV), which is probably affected by global changes with an increase in temperature and more rain (Chapter IV; Moreno and Perdomo, 2018; Eitzinger et al., 2014). The city has been established gradually with major steps linked to the conquest of flooded areas, the huge increase in population density and size with informal low-cost urbanization and housing and annexation of surrounding

municipalities (including part of *Usme*), and the recent policy for water management due to Public Health consideration and lifestyle issue (Peña-Guzmán et al., 2017; Rojas et al., 2015; Rojas, 2013). *Bogotá* currently stretches about 35 km from north to south and 20 km from west to east (**Chapter IV**), and has 20 districts.

Usme district (about 343 000 inhabitants in 2018 (Alcaldía Mayor de Bogotá, 2018)) has many neighbourhoods, including *Granada Sur*, *Quintas del Plan Social* and *La Aurora* neighbourhoods (having population densities of about 161, 161 and 227 inhabitants.ha⁻¹, respectively (SDP, 2018)), as well as the *Marichuela*, *Chunisa*, *G. Yomasa*, *Monteblanco*, *San Juan Bautista*, *Santa Librada*, *La Cabaña*, *Los Olivares*, *El Curubo* and *La Andrea* neighbourhoods (having population densities of about 227, 227, 227, 161, 227, 227, 227, 227, 227, and 227 inhabitants.ha⁻¹, respectively (SDP, 2018; Hospital de Usme ESE, 2010)) grouped in this study as the fictitious ‘Other’ neighbourhood. The *Granada Sur*, *Quintas del Plan Social*, *La Aurora* and ‘Other’ neighbourhoods are about 2 km north northeast of the *Doña Juana* landfill, where all municipal solid waste of *Bogotá* city and its surroundings are disposed (Caicedo et al., 2002), including soiled toilet papers and soiled diapers. The *Tunjuelito* River flows from south to north, west of these neighbourhoods and east of *Doña Juana* landfill; at the level of these neighbourhoods, this river is slightly contaminated by faecal indicators (**Chapter IV**). Further downstream (north) near its confluence with the *Bogotá* River, it receives raw wastewater from part of *Bogotá* inhabitants.

About 47% of the food items offered in the market place of *Bogotá* come from *Cundinamarca* department adjoining *Bogotá*; through farmers’ market or intermediaries (Eitzinger et al., 2014). Another 33% of the food passing through the *Corabastos* (*Central de Abastos*) wholesale market comes from the departments of *Boyacá*, *Tolima* and *Meta* (Eitzinger et al., 2014). Approximately 90% of the farmers in these departments are smallholders, producing roughly 70% of *Bogotá*’s food, owning or cultivating less than 20 ha of land (Montoya and Mondragón, 2010).

II.2. Survey content

133 households at different distances from the *Doña Juana* landfill in the *Quintas del Plan Social*, *Granada Sur*, *La Aurora* and ‘Other’ neighbourhoods (**Figure 1**) were visited from May to July 2018 by social workers from the *Asociación Integrada de Voluntarios Rurales y Urbanos* (ASIVRU) that bring together nurses, social workers, nursing technicians, psychologists and graduates in child pedagogy, one epidemiologist outside this association or

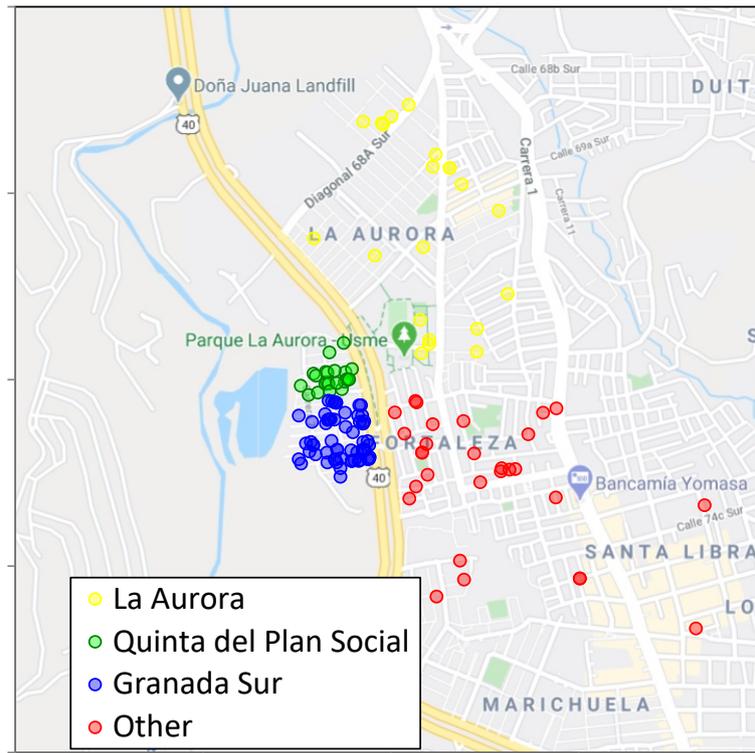


Figure 1: Approximate distribution of households interviewed in some of the neighbourhoods of Usme. (The *Doña Juana* landfill is between 1.7 and 2.7 km south southwest of the visited households).

one of the authors of this study (J.S. Jordan-Lozano) who asked an adult representative of each household questions about AGE and contextual elements likely to affect direct and indirect AGE transmissions. The interviewer wrote the answers to the questions on the investigation form. The questions successively addressed for the 1 year period preceding each survey:

- (i) The inhabitants of the household: their number and distribution by age group (<5 years, 5-18 years, 18-50 years, 50-75 years, and >75 years), the age of the person in charge, the neighbourhood and approximate GPS coordinates of the household, and the years already lived in it;
- (ii) Household socioeconomic conditions assessed by direct indicators – the professional condition and professional activity of people who meet the economic needs of the household –, and by indirect indicators about the maximum level of education achieved in home, the Health Insurance, the housing (its size in relation to the number of inhabitants, building materials, access to public services (drinking water, sewer system, electricity, gas, landline, internet, etc.), and the presence of pets;

- (iii) Household food practices: water consumed (tap water, bottled water) and food consumed (meat (beef, pork, chicken), fish, shellfish, vegetables, fruits), refrigerated storage of fresh food or not, food preparation (washing time, especially for vegetables and fruits eaten raw, peeling or not of fruit, cooking time of cooked food), frequency of consumption and the quantities consumed of water and various foods (per day or week) for all the household, but also specifically for young children (<5 years) and the elderly (>75 years) by focusing then on foods that are *a priori* most at risk (tap water, raw vegetables, raw fruits, raw meat, fish and/or seafood);
- (iv) Household hygiene practices: the frequency of hand washing, whether or not inhabitants wash their hands before eating, the preferred room for washing hands (bathroom, kitchen, laundry, etc.), the frequency of showers for inhabitants and its period during the day, the housework with the frequency of cleaning the main areas (bedrooms, living room, dining room, hallways, patios, bathrooms, toilets) and the type of products used (water, soap, disinfectant, etc.), the frequency of household waste disposal;
- (v) Household AGE cases by age group over the past 12 months – AGE cases being restricted in this work to diarrhoea cases with more than two bowel movements per day for more than two days, with or without other clinical symptoms such as stomach pain, nausea, vomiting or fever –, and health practices to treat these cases, with the places where the patients went (naturopathic centre, pharmacy, health centre allowing to consult a physician, a clinic or an hospital), possibly the names of home remedies, naturopathic remedies and/or drugs provided, and cases of hospitalized patients.

Each investigation form could thus contain up to 75 answers relating to the household visited which corresponded to 5, 12, 34, 10 and 14 questions about household 'inhabitants', 'socioeconomic conditions', 'food practices', 'hygiene practices' and 'AGE ad health practices', respectively. The investigations respected the anonymity of each household visited.

II.3. Survey analysis

While the answers to some questions were numerical values, the answers to most questions were of a qualitative types (e.g. yes/no, a job category, the access to some public services) or needed to be synthesized when they contained several pieces of information (e.g. the number of inhabitants sleeping in each room of the household). Consequently, we defined indexes which varied monotonously with the potential pressure of the variable considered on AGE (e.g. the number of pet types, the occupancy level of the bedrooms) and/or with other

indirectly linked variables (e.g. access to landline, internet, satellite dish and cable TV). For each of the 75 questions on the survey form, the up to 133 responses or index values were first assessed, so as to eliminate questions deemed non-discriminatory in this work when at least 95% of responses were identical.

Principal Component Analyses (PCAs) were then carried out separately for some and/or for all of the variables kept in each of the 'inhabitants', 'socioeconomic conditions', 'food practices', 'hygiene practices' and 'AGE and health practices' headings. This step made it possible on the one hand to identify factors discriminating the neighbourhoods of the city, on the other hand to identify groups of variables strongly correlated with each other. This last aspect allowed reducing the number of variables representative of each of the headings to carry out a PCA on a set of variables representative of all the headings mentioned above, in order to identify factors/variables correlated with AGEs and try understanding the underlying reasons. From a technical point of view, the PCAs were carried out on renormalized variables in three successive stages: first the analysis of the matrix of linear correlation coefficients, second the analysis of the representativeness of the plane 1-2 and of the projected vectors of the variables in this plane, and third the analysis of the projected households in this plane by distinguishing their neighbourhoods. In addition to PCA analyses, certain correlations between variables were described by histograms using one of the variables as abscissa and the other as serial number.

II.4. Model of AGE transmission

We defined p as the (unique) probability that an inhabitant of a household was infected outside this household during the 12 months preceding each survey, and then developed an AGE. We defined healthy households as households without AGE case during this 12 months period. The probability that a household with n inhabitants was healthy can be estimated by the following equation:

$$1 - P_{\text{out}}(n) = (1 - p)^n \quad (1)$$

where $P_{\text{out}}(n)$ is the probability that at least one of the n inhabitants of this household developed an AGE during the 12 months. Variations in the proportions of healthy households are presented in [Figure 2](#) for $n \leq 9$ and $0.05 \leq p \leq 0.3$, knowing that there were no more than 9 inhabitants per household surveyed and that p seemed to be close to 0.15 in this study. [Figure 2](#) shows that variations in p values higher than about 0.05 led to greatly differing

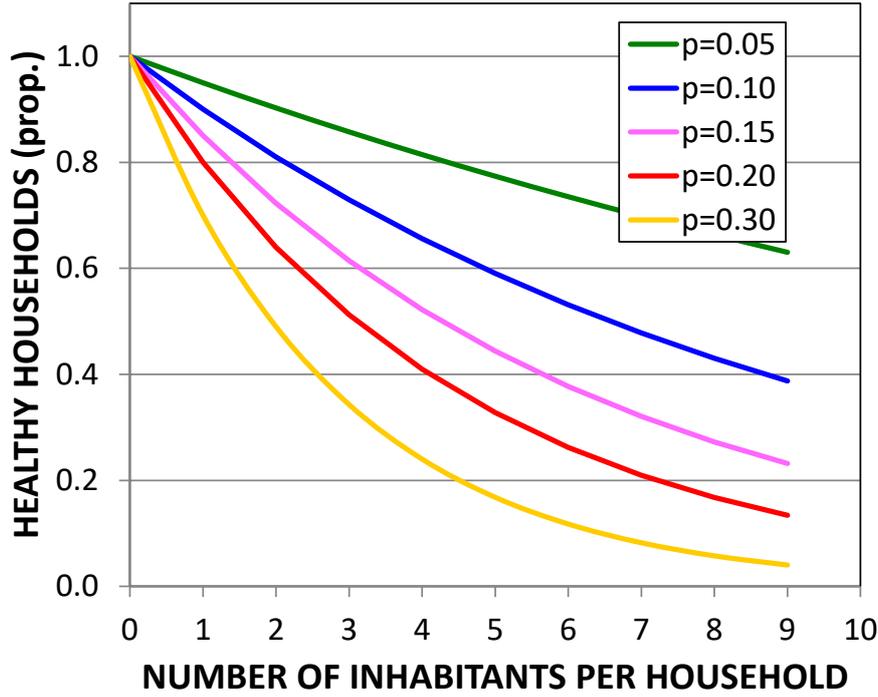


Figure 2: Simulated variations in the proportion of healthy households (i.e. without any AGE case) as a function of the number of inhabitants per household for different values of the probability p that an inhabitant of a household was infected outside this household.

proportions of healthy households, especially for those with 3 to 5 inhabitants, which correspond to a large proportion of the households surveyed in this study. In the neighbourhoods surveyed in the district of *Usme*, p was estimated by fitting the simulated proportion of healthy households to the proportion of healthy households calculated from the survey; considering either households in all the neighbourhoods surveyed, or households in only one of these neighbourhoods.

In addition, knowing p , it is possible to estimate the probability $P'(k|n)$ that exactly k inhabitants among the n inhabitants of a household were infected outside this household during the **12** months preceding the survey for this household with $k \leq n$:

$$P'(k|n) = \binom{n}{k} \times p^k \times (1 - p)^{n-k} \quad (2)$$

Of course, we have:

$$P'(0|n) = 1 - P_{\text{out}}(n) \quad (3)$$

We defined p' as the (unique) probability that a sick inhabitant transmitted AGE to a healthy inhabitant of the same household during the 12 months period preceding the survey. For a household with n inhabitants, k of whom having developed AGE following an infection

outside the household, the probability that k' other inhabitants developed AGE after direct transmission from a sick inhabitants of the same household can then be estimated by making the following assumptions:

- assuming k_1 AGE cases in this household with $k \leq k_1 < n$, the probability $P_{in}(k_1)$ for a healthy inhabitant to be infected inside the household by one of the k_1 sick inhabitants and then develop AGE is:

$$P_{in}(k_1) = 1 - (1 - p')^{k_1} \quad (4)$$

- whatever the value of k with $0 \leq k < n$, the probability $P''(k'=0 | n, k)$ that there are no infection inside of the $n-k$ healthy inhabitants from the k AGE cases ($k'=0$) is then:

$$P''(k' = 0 | n, k) = 1 - (1 - P_{in}(k))^{n-k} \quad (5)$$

- whatever the value of k with $0 \leq k < n$, the probability $P''(k'=n-k | n, k)$ that the k AGE cases caused by outside infections led to inside infection of the other $n-k$ inhabitants is:

$$P''(k' = n - k | n, k) = \prod_{k_1=k}^{n-1} P_{in}(k_1) \quad (6)$$

- for k' neither equal to 0 nor to $n-k$, the probability $P''(k' | n, k)$ that the k AGE cases resulting from outside infections led to k' additional AGE cases from inside infections can then be estimated iteratively using the following equation:

$$P''(k' | n, k) = \frac{\left(P''(k' | n - 1, k) \times (1 - P_{in}(k + k')) \right) + \left(P''(k' - 1 | n - 1, k) \times P_{in}(k + k' - 1) \right)}{\quad} \quad (7)$$

The same estimates of $P''(k' | n, k)$ could have been obtained by considering possible arrangements of household inhabitants with first the k AGE cases resulting from infections outside the household, and second all the possible arrangements of k' AGE cases resulting from infections inside the household and $n-(k+k')$ inhabitants that remained healthy during the 12 months period preceding the survey (see **Figure 3** for an example).

Then, the probability $P(K|n)$ that K inhabitants among the n inhabitants of a household were sick in could be estimated by the following equation:

$$P(K|n) = \sum_{k=0}^K P'(k|n) \times P''(k' = K - k | n, k) \quad (8)$$

Variations in the proportions of healthy households are presented in **Figure 4** for $3 \leq n \leq 5$, for $p=0.15$, and for $0.0 \leq p' \leq 0.3$, knowing that p' seems to be close to 0.15 for this study. **Figure 4** shows that an increase in p' did not affect the proportion of households with no AGE sick people, but increase the number of AGE cases in non-healthy households. In the neighbourhoods surveyed in the district of *Usme*, p' was estimated by fitting the simulated

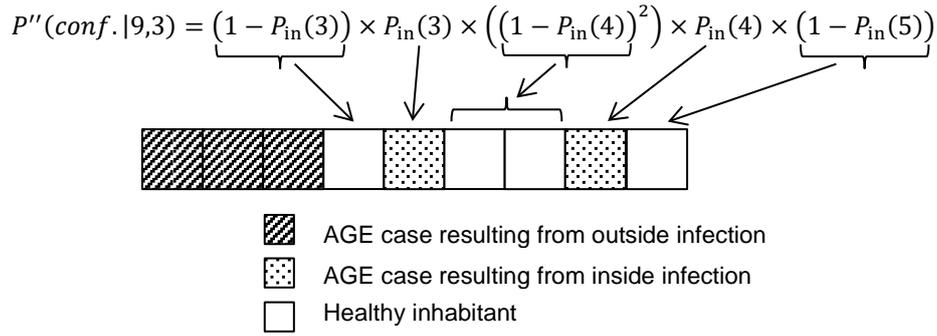


Figure 3: One of the 15 possible arrangements that have to be taken into account to calculate $P''(2|9, 3)$ for the case of an household with $n=9$ inhabitants, $k=3$ inhabitants infected outside and developing an AGE, and $k'=2$ inhabitants infected within the household.

proportion of sick people to the proportion of AGE cases calculated from the survey; considering either households in all the neighbourhoods surveyed, or households in only one of these neighbourhoods.

Note that this model did neither offer the possibility of asymptomatic infected peoples as observed for several AGE pathogens including norovirus (Teunis et al., 2015), nor take into account a variability in p and p' , including variations in p and/or p' with AGE aetiology, and variations with people age that may result from acquired immunity (Lewnard et al., 2017).

In order to assess the uncertainty on p and p' estimates, we performed 20 random simulations of AGE cases for exactly the same household configuration (number of households and number of inhabitants in each household) as those surveyed in *La Aurora*, *Quintas del Plan Social*, *Granada Sur* and 'Other' neighbourhoods for $p=0.15$ and $p'=0.15$, these values being very close to the values estimated from the surveys. For each of the 20 achievements of AGE cases in all of these households, we then estimated p and p' for each of the four neighbourhoods and for all the neighbourhoods considered together. The estimated values were dispersed with more dispersion on p' than on p (Figure 5 and Table 1); it was minimum for all the neighbourhoods considered together, and maximum for the neighbourhoods with the smallest number of households surveyed. The dispersion decreased in the order *Quintas del Plan Social* (23 households) \approx *La Aurora* (21) > 'Other' (29) > *Granada Sur* (60) > All neighbourhoods (133). Considering the 20 simulations of AGE transmission in the 133 households surveyed, the mean, the first decile and the ninth decile of the difference between the maximum p estimate and the minimum p estimate for neighbourhoods were 0.087, 0.047 and 0.134, respectively; similarly, the mean, the first decile and the ninth decile of the difference between the maximum p' estimate and the

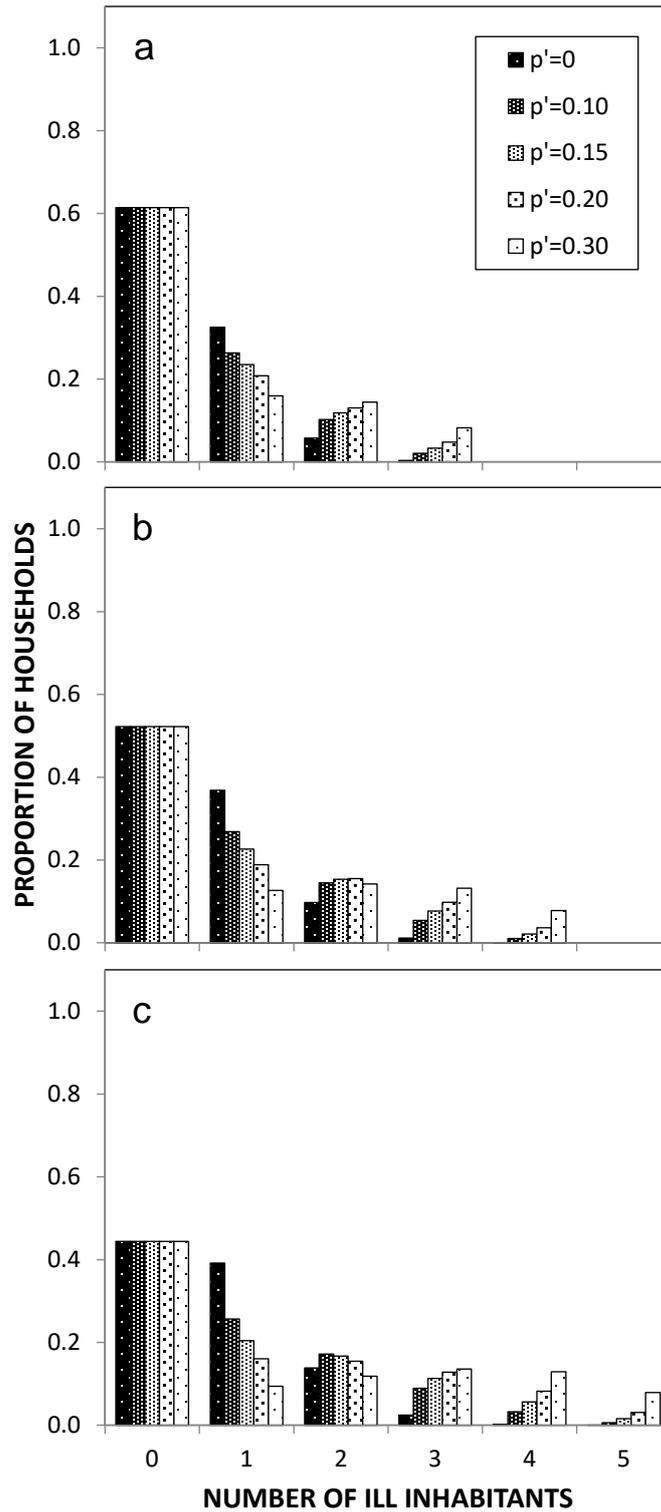


Figure 4: Average distribution of the number of inhabitants suffering from AGE within households having a 3 (a), 4 (b) and 5 (c) inhabitants for $p=0.15$ and $0.0 \leq p' \leq 0.3$.

minimum p' estimate for neighbourhoods were 0.129, 0.068 and 0.218, respectively. Although the hypotheses used to build this model can be discussed (in particular the fact that AGE transmission is a random process independent of households and people in the same

neighbourhood, or even across all neighbourhoods), these values can be regarded as indications on the minimum differences p or p' values between the neighbourhoods surveyed that can be considered significant.

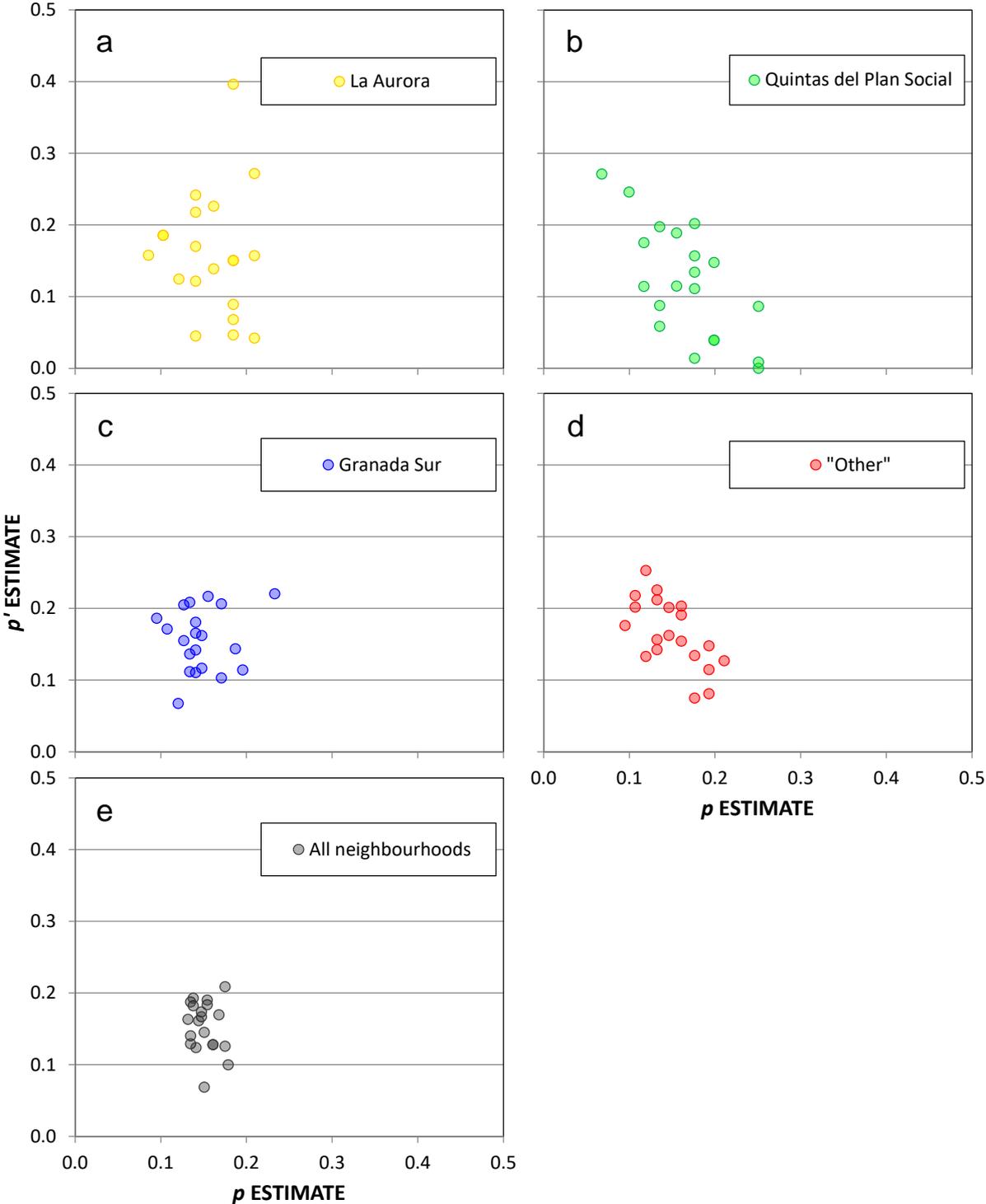


Figure 5: p and p' estimates obtained for the 20 simulations of AGE transmission in *La Aurora* (a), *Quintas del Plan Social* (b), *Granada Sur* (c) and ‘Other’ (d) neighbourhoods, as well as in all these four neighbourhoods together (e).

	p estimate			p' estimate		
	D_1	Average	D_9	D_1	Average	D_9
All neighbourhoods	.135	.151	.175	.112	.153	.191
<i>La Aurora</i>	.103	.159	.209	.046	.159	.257
<i>Quintas del Plan Social</i>	.108	.168	.255	.011	.120	.224
<i>Granada Sur</i>	.114	.148	.191	.107	.156	.213
'Other'	.107	.150	.193	.098	.165	.222

Table 1: First decile (D_1), average and ninth decile (D_9) of p and p' estimates for the 20 simulations of AGE transmissions in the neighbourhoods surveyed.

III. Results and Discussion

III.1. Preliminary analysis

Considering that the questions for which at least 95% of the answers were identical were not relevant for this study, we excluded 10 of the 75 questions from the survey form. In practice, we worked on the answers to 5, 9, 31, 6 and 14 questions relating to 'inhabitants', 'socio-economic conditions', 'food practices', 'hygiene practices', and 'AGE cases and health practices', respectively.

III.2. Variations in AGE cases with age, household composition and neighbourhood

The 133 households surveyed corresponded to 525 inhabitants, 114 of whom suffered from AGE during the 12 months preceding the survey. This corresponded to 22% of the surveyed population, which was lower than the proportions estimated elsewhere (e.g. in France ([Van Cauteren et al., 2012](#)) and in the US ([Lively et al., 2018](#))). However, the study only relied on a small sample of the Colombian population for a single year. Moreover, this proportion was based on diarrhea cases; other more encompassing definitions consider AGE all cases with diarrheal or vomiting symptoms. In France, [Tesson et al. \(2019\)](#) found that prescriptions of antiemetic only were approximately 0.5 to 1 times the prescriptions with anti-diarrhea alone or with antiemetic, suggesting that taking into account cases with vomiting symptom without diarrheal symptom would have increased the fraction of the population that suffered from AGE during the 12 months that preceded their household survey.

The proportion of AGE cases (based on diarrheal symptoms) decreased with increasing

the age (**Table 2**). The decrease of the proportion of AGE cases with increasing age is similar in France ([Van Cauteren et al., 2012](#)), but we must not forget that the impact of AGE is generally much more serious in the elderly ([Ansaldi, 2019](#)).

Age group	< 5 years	5-75 years	> 75 years
Total	114	371	40
AGE cases	34	77	3
% of sick people	30%	21%	8%

Table 2: Variation in the proportion of AGE cases with the age group for the 525 inhabitants living in the 133 households surveyed in this study.

Considering simultaneously all the neighbourhoods, this dependency may explain why the proportion of households with AGE cases decreased in the order ‘<5 years + 5-75 years’ > ‘5-75 years’ \approx ‘<5 years + 5-75 years + >75 years’ > ‘5-75 years + >75 years’ > ‘>75 years’ (**Table 3**). It was not possible to check the same relationship for each neighbourhood due to the too small number of households in each category. However, it seemed that the proportion of households with ill inhabitants *a priori* depends on the neighbourhood in the order *La Aurora* < *Quintas del Plan Social* < *Granada Sur* \approx ‘Other’.

p was estimated by fitting the simulated proportion of healthy households to the proportion of healthy households calculated from the survey; considering either households in all the neighbourhoods surveyed (**Figure 6**) or households in only one of these neighbourhoods (**Table 4**). Similarly, p' was estimated by fitting the simulated proportion of

Age groups	All households	<5 + 5-75 years	5-75 years only	<5 + 5-75 + >75 years	5-75 + >75 years	>75 years only
All neighbourhoods	45% (132)	48% (63)	45% (31)	43% (28)	33% (9)	0% (1)
<i>La Aurora</i>	33% (21)	30% (10)	0	0	0	
<i>Quintas del Plan Social</i>	41% (22)					
<i>Granada Sur</i>	48% (60)	54% (24)	48% (21)	50% (10)		
“Other”	48% (29)	43% (21)				

Table 3: Proportions of households with AGE cases according to neighbourhoods and age groups cohabiting in each household. The numbers of households with and without sick inhabitants are in parentheses. (Values specific to neighbourhoods were reported only when the total number of households considered were greater or equal to 10).

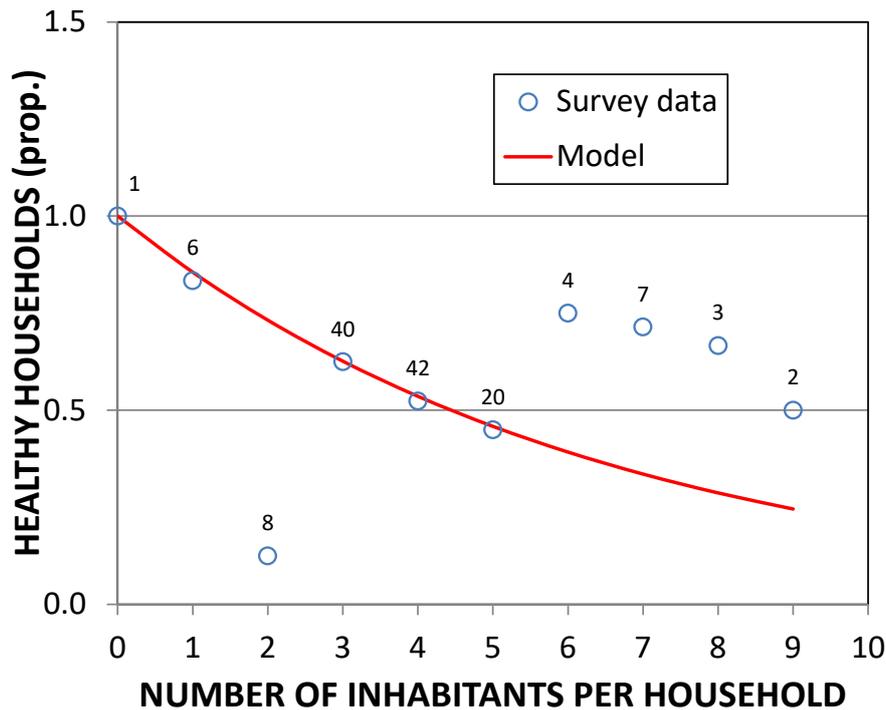


Figure 6: Proportion of healthy households (i.e. without AGE case) as a function of the number of inhabitants per household. The “Survey data” are for all the 133 investigated households. The point labels are the numbers of households contributing to each point. Simulations were performed for $p=0.144$.

	All neighbourhoods	<i>La Aurora</i>	<i>Quintas del Plan Social</i>	<i>Granada Sur</i>	“Other”
p	0.144	0.103	0.136	0.163	0.146
p'	0.144	0.155	0.198	0.141	0.110

Table 4: p and p' estimates for all the neighbourhoods considered together and for each of these neighbourhoods.

ill cases to the proportion of ill cases calculated from the survey; considering either households in all the neighbourhoods surveyed (**Figure 7**) or households in only one of these neighbourhoods (**Table 4**). For all the neighbourhoods considered simultaneously, the simulated proportion of healthy households agreed well with the survey for households with 3, 4 or 5 inhabitants (**Figure 6**) that represent 77% of surveyed households (102/133). There was *a priori* no relationship between the estimated p and p' values (**Figure 8**). Moreover, the difference between the maximum and minimum p values (0.060), as well as the difference between the maximum and minimum p' values (0.088) suggested that no difference in AGE

cases between the four neighbourhoods could be detected from the survey.

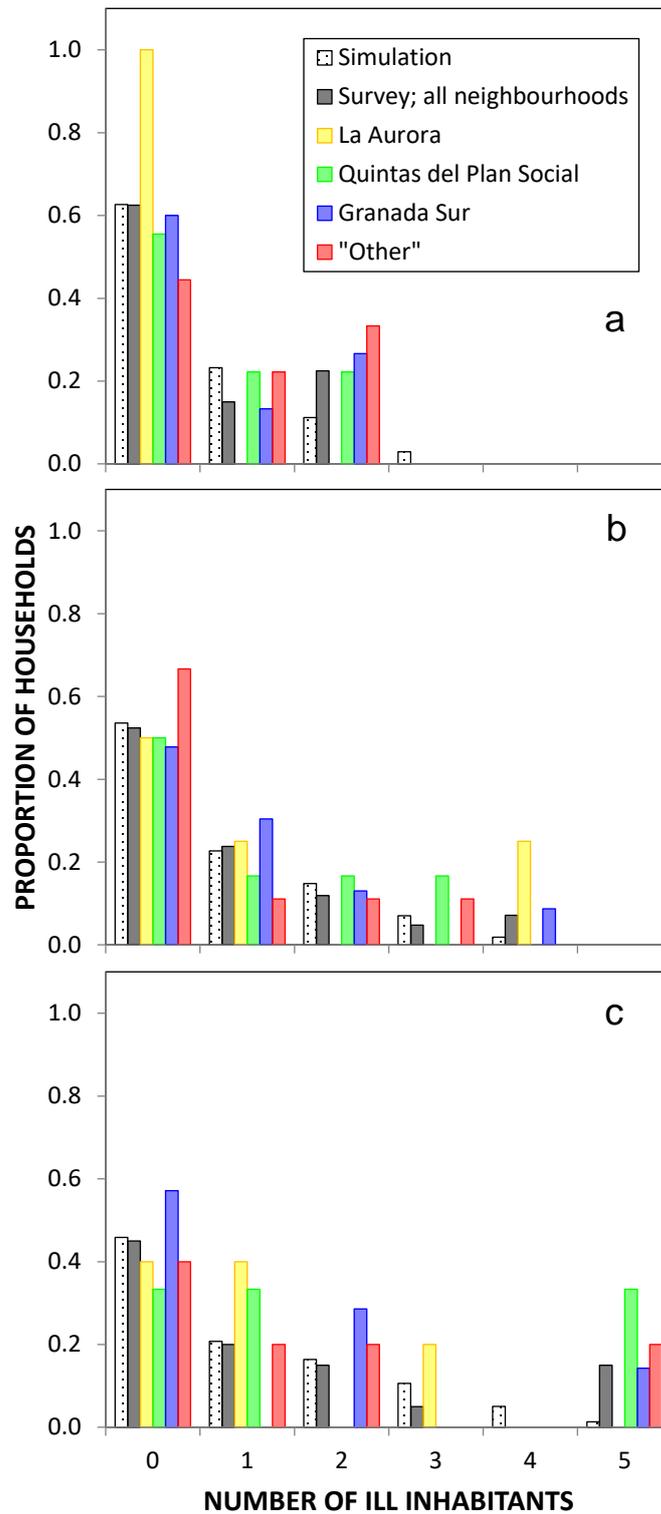


Figure 7: Proportion of households with 0 to 5 sick people for households with 3 (a), 4 (b) and 5 (c) inhabitants in *La Aurora*, *Quintas del Plan Social*, *Granada Sur* and/or 'Other' neighbourhoods. Simulations were performed for $p=0.144$ and $p'=0.144$.

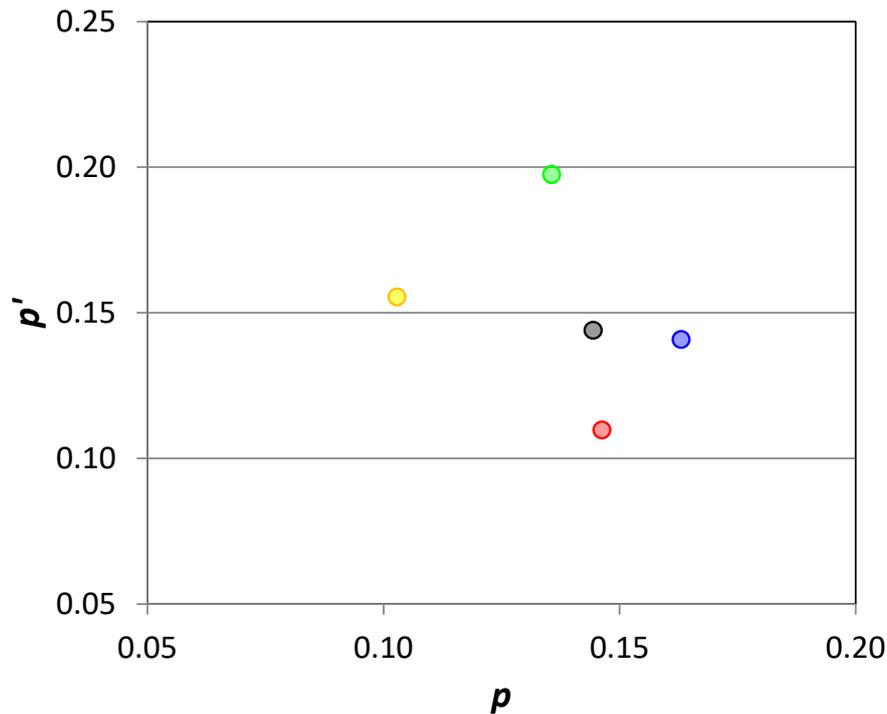


Figure 8: p and p' estimates for all the neighbourhoods considered together and for each of these neighbourhoods.

III.3. Analysis specific to thematic headings and combining thematic headings

In this sub-section, we only present the main results obtained by PCA and by additional analyzes on each of the “inhabitants”, “socio-economic conditions”, “food practices”, “hygiene practices” and “AGE and health practices” headings. The analysis turned out to be more complex than expected, with certainly at least two underlying reasons: (1) the limited number of surveys (133) and (2) correlations between variables that should not be interpreted as relationships between cause and effect, with complex interdependencies: for example, positive correlations between the consumption of raw fruits and vegetables and hygiene practices raw may affect the correlation between the consumption of raw fruits and vegetables and AGE cases. In some cases, hypotheses suggested by certain correlations could be invalidated by further analysis: for example, the lower presence of pets in *La Aurora* neighbourhood is in no way related to the lower frequency. of AGE in this neighbourhood.

Household inhabitants: The number of inhabitants per household varied between 1 and 9 (Figure 9), but households with 3, 4 and 5 inhabitants corresponded to 77% of all the

households visited (102/133).

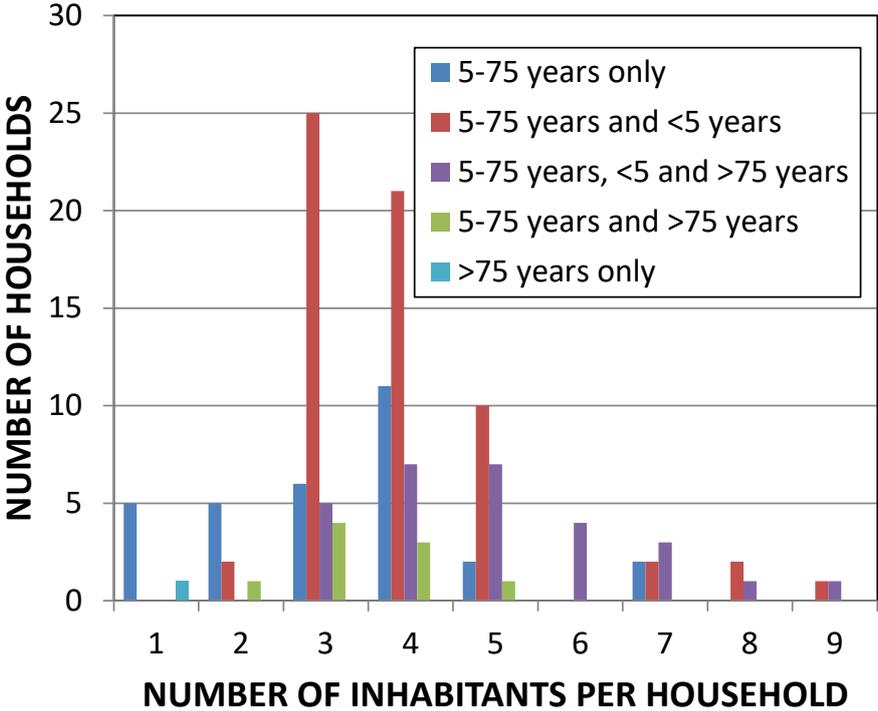


Figure 9: Number of households as a function of their total number of inhabitants for households with or without very young children or very old elderly people.

There was obviously a strong correlation between the total number of inhabitants and the number of 5-75 years inhabitants per household ($R=0.83^{**}$), since this age group corresponded to most of the population. Linear correlations were also statistically significant between the total number of inhabitants per household and either the number of young children ($R=0.44^*$) or the number of elderly people ($R=0.26^*$) per household.

By contrast, the geographic distribution of surveyed households seemed apparently independent to the number of household inhabitants, although (i) *Quintas del Plan Social* neighbourhood has a lower mean number of inhabitants per household, (ii) *La Aurora* and “Other” neighbourhoods appeared younger, as reflected by the mean ages of the household head and the mean numbers of children <5 years per household, and (iii) *Quintas del Plan Social* and *La Aurora* neighbourhoods had simultaneously slightly more people >7 years than *Granada Sur* and “Other” neighbourhoods (**Table 5**).

Neighbourhood	Head age	Inhabitants	< 5 years	5-75 years	> 75 years
			----- Number per household -----		
<i>La Aurora</i>	37.4	4.12	1.00	2.73	0.38
<i>Quintas del Plan Social</i>	41.5	3.57	0.78	2.35	0.43
<i>Granada Sur</i>	42	3.93	0.73	2.92	0.28
Other	34.8	4.17	1.08	2.96	0.13

Table 5: Mean total number of inhabitants and mean number of inhabitants in different age groups in *La Aurora*, *Quintas del Plan Social*, *Granada Sur* and Other neighbourhoods.

Household socioeconomic conditions: PCA showed that the “flexibility in bedroom occupancy” was negatively correlated with the total number of household inhabitants and to a lesser extent positively correlated to the number of pet types (dogs, cats, birds ...) in home (**Figure 10a**). These three variables seemed to be not correlated to other variables in the PCA in **Figure 10**, except a small positive correlation between the total number of inhabitants per household and the Health Insurance ($R=0.20^*$). Health Insurance, the level of initial formation, the professional status and the salary range were also positively correlated to each other (**Figure 10a**). However, the proportion of AGE cases in households was not correlated with the previously mentioned variables (pink arrow in **Figure 10a**). The projection of the households on the PCA plane 1-2 apparently did not show differences between *La Aurora*, *Quintas del Plan Social*, *Granada Sur* and “Other” neighbourhoods, although the projections of households in *La Aurora* neighbourhood seemed to be more abundant in the bottom left quarter of **Figure 10b**. The average projections of the households confirmed the apparent differences between *La Aurora* and “Other” neighbourhoods on the one hand, and *Quintas del Plan Social* and *Granada Sur* neighbourhoods on the Other hand (**Figure 10a**). Average values for each of these four neighbourhoods confirmed first that *La Aurora* and “Other” neighbourhoods had higher average number of total inhabitants than the other two neighbourhoods (**Table 5**), and second that *La Aurora* neighbourhood had higher salaries than the other three neighbourhoods (**Table 6**). Simultaneously, we note that the number of animal species per household decreased with the increase in the number of young children (**result not shown**), and that the number of animal types per household in *La Aurora* neighbourhood was lower than in the other three neighbourhoods (**Figure 11**). As *La Aurora* neighbourhood seemed to have both a lower proportion of household with AGE cases and less animals per household than other neighbourhoods (**Table 3**, **Table 6** and **Figure 11**), we checked whether animals could explain higher level of AGE: considering all the surveyed households, we found that 47% of the households with no animals had AGE cases, while 42% of the households with animals had AGE cases.

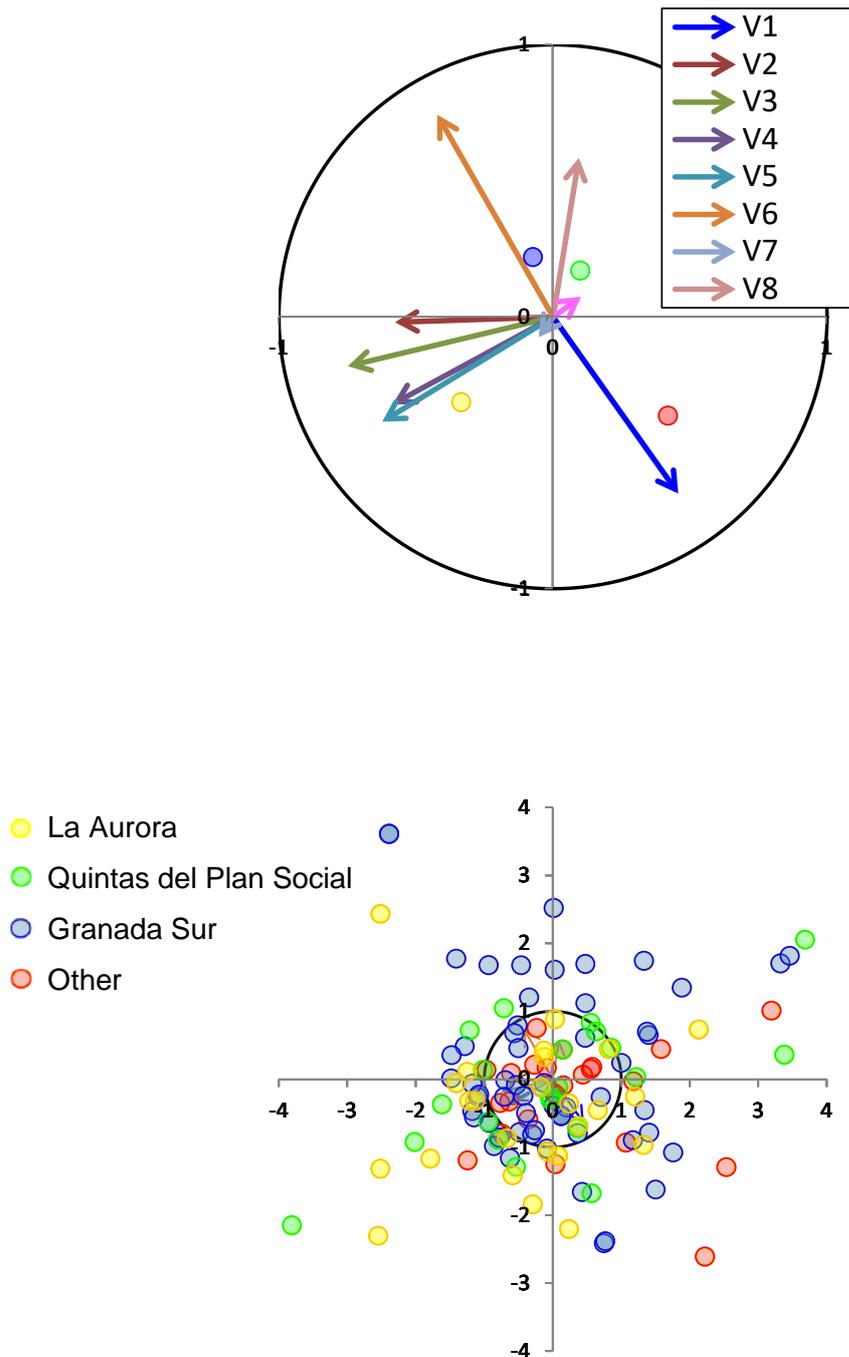


Figure 10a-b: PCA for socioeconomic conditions with projections of the variables on the factor plane 1-2 (a) and projections of the households on the factor plane 1-2 (b). (V1: number of inhabitants; V2: health protection; V3: initial formation; V4: professional status; V5: salary range; V6: flexibility in bedroom occupancy; V7: access to landline, internet, satellite dish and cable television; V8: pet species. Axis 1 and 2 explain 25.0% and 18.4% of the variability, respectively). The proportion of patients per household is an additional variable not taken into account in the PCA calculations (pink arrow in (a)).

Neighbourhood	Salary index	Access to “public services” index	Animal index
	----- Index values without unit -----		
<i>La Aurora</i>	3.00	2.88	0.23
<i>Quintas del Plan Social</i>	2.70	2.83	0.61
<i>Granada Sur</i>	2.58	2.75	0.77
Other	2.58	2.71	0.50

Table 6: Indexes related to Salary range, access to “public services” (landline, internet ...), and number of animal species in *La Aurora*, *Quintas del Plan Social*, *Granada Sur* and “Other” neighbourhoods.

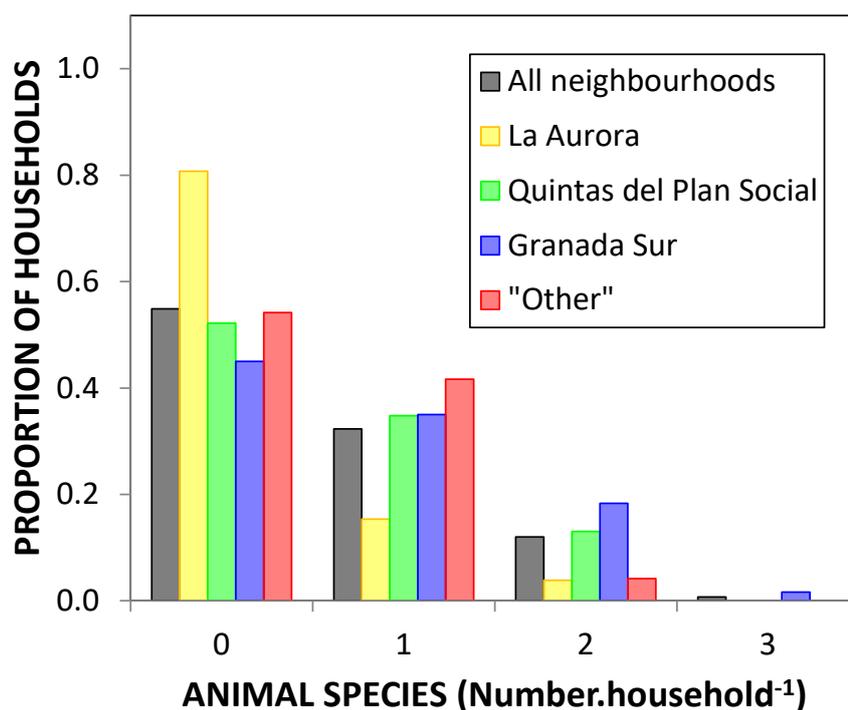


Figure 11: Distribution of the number of animal types per household as a function of the neighbourhood.

Food practices: 9 households (<7% of the surveyed households) did not have a refrigerator to store fresh foods (vegetables, fruits, meats, sausages) at low temperature; 1, 5 and 3 of these households were in *La Aurora*, *Granada Sur* and "Other" neighbourhoods, respectively. No correlation was statistically significant between the AGE cases did not appear correlated with variables describing food individual practices (**Figure 12**).

		Number of inhabitants <5 years	Number of inhabitants 5-75 years	Number of inhabitants >75 years	Index of salary range	Frequency of beef consumption	Frequency of pork consumption	Frequency of chicken consumption	Frequency of fish consumption	Frequency of shellfish consumption	Frequency of raw vegetable consumption	Frequency of raw and unpeeled fruit cons.
		1	2	3	4	5	6	7	8	9	10	11
Number of inhabitants <5 years	1	1.000	-0.031	0.081	-0.097	-0.111	-0.165	-0.042	-0.115	0.084	-0.161	-0.149
Number of inhabitants 5-75 years	2		1.000	-0.094	0.034	-0.022	0.005	-0.005	-0.104	-0.090	0.049	-0.049
Number of inhabitants >75 years	3			1.000	-0.052	0.022	0.143	0.159	0.157	0.142	0.113	0.130
Index of salary range	4				1.000	0.015	0.094	-0.065	-0.002	0.088	0.110	0.213
Frequency of beef consumption	5					1.000	0.252	0.285	0.233	-0.001	0.217	0.291
Frequency of pork consumption	6						1.000	0.003	0.026	0.150	0.129	0.276
Frequency of chicken consumption	7							1.000	0.155	0.030	0.201	0.249
Frequency of fish consumption	8								1.000	0.268	0.126	0.124
Frequency of shellfish consumption	9									1.000	-0.124	0.037
Frequency of raw vegetable consumption	10										1.000	0.410
Frequency of raw and unpeeled fruit cons.	11											1.000

Figure 12: Upper half of the correlation matrix between 11 variables (direct answer or their conversion into indexes).

Hygiene practices: PCA showed that the presence of elderly people (>75 years) in households was positively correlated with the time used to rinse fruits and vegetables eaten ($R=0.364^{**}$) raw or meat, fish and shellfish ($R=0.179^*$) (results not shown); the variability between households having the same number of elderly people >75 years is high, as it may be illustrated by **Figure 13**. These two rinsing indexes were of course correlated with each other ($R=0.494^{**}$). By contrast, these two rinsing indexes were not correlated with the index characterizing the preference of consumption of peeled and/or unpeeled fruits. A more complete analysis carried out on all the surveyed households and separately on each of the four neighbourhoods by distinguishing households on the presence of children <5 years, inhabitants 5-75 years and elderly persons >75 years enabled to confirm that the indexes characterising the duration of fresh produce rinsing closely increased with the presence of elderly persons >75 years (**Table 7**). These “rinsing” indexes seemed to be *a priori* not significantly correlated with all other variables characterizing ‘household inhabitants’, ‘household socioeconomic conditions’ and ‘household food practices’. Surprisingly (!) the index characterizing household preference for eating peeled and/or unpeeled fruits was correlated with the index characterizing the frequency of raw vegetable consumption. In the

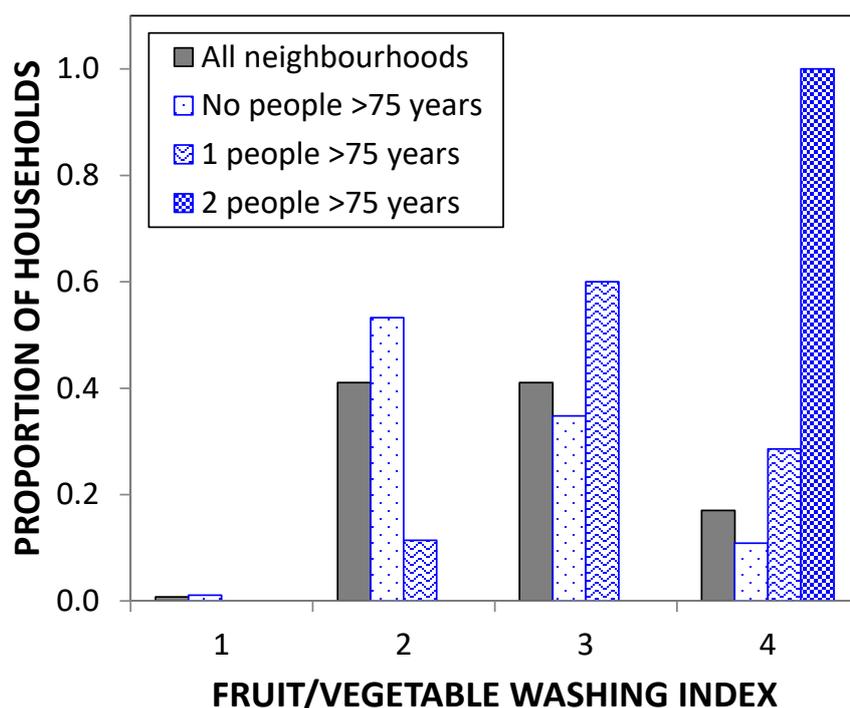


Figure 13: Variability in the index for the rinsing time of fruit/vegetable eaten raw (1: never; 2: 1 min; 3: 2 min; 4: 4 min) as a function of the number of people >75 years per household. (The numbers of households with 0, 1 or 2 >75 years are displayed next to the histogram bars).

Age groups	Neighbourhood				
	All	La Aurora	Quintas del Plan Social	Granada Sur	Other
5-75 years only	2.50 (31)	2.50 (2)	2.50 (4)	2.43 (21)	3.00 (4)
<5 years + 5-75 years	2.46 (68)	2.57 (14)	2.50 (8)	2.33 (24)	2.53 (17)
<5 years + 5-75 years + >75 years	3.14 (28)	3.43 (7)	3.50 (8)	2.70 (10)	3.00 (3)
5-75 years + >75 years	3.11 (9)	3.67 (3)	2.50 (2)	3.00 (4)	-
>75 years only	3.00 (1)	-	-	3.00 (1)	-

Table 7: Average index characterizing the time for rinsing fruit and raw vegetable before consumption (1: no rinsing; 2: 1 min; 3: 2 min; 4: 4 min) as function of *Usme* neighbourhood and the age groups living in the households. (Numbers in parenthesis are the number of households that allowed to perform these calculations; in the absence of households falling into a given category, the table cell was filled with a "-").

surveys carried out, the total percentages of households with elderly people > 75 years in *Quintas del Plan Social* and *La Aurora* neighbourhoods (45.5% and 38.5%, respectively) are much higher than those in the districts of *Granada Sur* and "Other" (25.0% and 12.5%, respectively). The rinsing time indexes were not correlated with the corresponding indexes for the frequency of food consumption (results not shown).

IV. Conclusion

Unfortunately, no simple relationship has emerged between AGE cases per household on the one hand, and the socioeconomic context and individual (food, hygiene and health) practices on the other. This does not mean that there is no cause and effect relationship but that the links between household context and AGE, if they exist, have not been identified by the surveys, due to their weak number (133) and multiple proximal and distal variables affecting direct and indirect AGE transmission.

In its current state, the study has enabled us to highlight certain relationships that will probably remain true in the next few years for *Bogotá* and its surroundings: number of inhabitants and flexibility on the bedrooms, absence of significant impact of pets on AGE transmissions, better hygiene during the preparation of food at home in the presence of elderly people.... This work has also allowed us to propose a model that should be seen as an analytical tool and not as a realistic model because it greatly simplifies reality. Used to stochastically simulate the transmission of AGE from the outside to the interior of the households, and between inhabitants within households, this model has shown how much the randomness of infections can ultimately affect the estimated values of certain parameters. (p and p' in this study).

The simulations confirm the need for much higher numbers of surveys to reduce uncertainties and to be able to highlight certain relationships. An alternative would be to work on critical episodes in epidemic terms (several cases of AGE within a home, a neighborhood or a community (restaurant, hospital, canteen) for example), but this type of studies would probably not address the same questions.

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Chapter VI:

Discussion – General Conclusion

I. Introduction: reminders on the main objectives of the PhD

The general objective of this PhD was to evaluate the flows of certain human enteric pathogens in the environment near *Bogotá* (Colombia) and to relate these flows to cases of AGEs in the population. In practice, this objective was divided into three sub-objectives: (i) the development of a culture method for human norovirus (cf. **Chapter III**) because it is one of the main pathogens of humans involved in AGEs, (ii) the characterization of the contaminations of water, leafy-vegetables – using chard as a model of leafy vegetable that can be eaten raw – and air by various human enteric pathogens (virus, bacteria and unicellular parasite) as well as by markers of faecal contamination in *Bogotá* (Colombia) and its surroundings (cf. **Chapter IV**), and (iii) the assessment of the existence of relationships between cases of AGEs on the one hand, and standards of living and individual practices (food, hygiene and health) on the other hand (cf. **Chapter V**).

II. Main findings of the PhD, discussion

II.1. Contamination of water, food and air

Water contamination: The quality of the water greatly varied with its origin with, *a priori*, groundwater upstream of *Bogotá* little or not contaminated by faecal indicators and pathogens, and surface water slightly to heavily contaminated depending on its location in relation to the discharge points of wastewater of *Bogotá* and nearby towns and depending on the treatment before discharge. Wastewater was generally discharged without treatment or after primary treatment only. Thus, the irrigation water at *La Ramada* near *Mosquera* was heavily contaminated with *E. coli* unlike the groundwater supplying *Cota*; the contamination of the surface water used in *Muchuelo Alto* was intermediate. Contamination of surface water by faecal indicators in our work was of the same order of magnitude as that obtained in *La Ramada* by [Heliodoro and Bustos-López \(2018\)](#), and in the *Tunjuelito* river by the *Corporación Autónoma Regional (CAR, 2019)* and the *Secretaría Distrital de Ambiente (SDA, 2019)*. Other authors who used *E. coli* as an indicator of faecal contamination in surface water receiving wastewater found similar results ([Sánchez-Alfonso et al., 2020](#); [Venegas et al. ., 2015](#)), *E. coli* concentration being much higher in wastewater ([Haramoto et al., 2006b](#); [Garcia-Armisen and Servais, 2004](#); [Torrella et al., 2003](#)) unlike that in water

sources (Venegas et al., 2015) or in rivers upstream of any wastewater discharge (Muirhead and Meenken, 2018; Widmer et al., 2013).

By contrast, the human enteric pathogens monitored have not been systematically detected in water with a high load in faecal indicators, at least by molecular method (RT-qPCR or qPCR): for example, noroviruses were detected that in 7%, 23% and 0% of water samples from the *Tunjuelito* river (upstream of the large wastewater discharges from *Bogotá*) (2/30), from the storm-water collector flowing into this river and sampled in our work (receiving wastewater from some nearby houses) (7/30) and from leachate from the *Doña Juana* landfill treated by a specific wastewater treatment plant near this landfill (0/4), respectively. In addition, the norovirus concentrations found in our work were low, whereas these viruses are almost systematically found in rivers, as soon as they receive wastewater (Prevost et al., 2015; Lodder et al., 2010; Hamza et al., 2014; Hamza et al., 2009; Haramoto et al., 2005). Some reasons may be invoked to explain the few positive samples and the low concentrations in positive samples: the small volume of water samples (3 L only against 200 to 600 L in Lodder et al. (2010)) resulting in a higher detection threshold (of the order of 15 GC.L⁻¹ for the water from the river and the collector, and 7 GC.L⁻¹ for the leachates), the possible inhibitions in qPCR, and pre-filtration to retain coarse particles (Cucarella and Renman, 2009) using polyamide filters with 0.2 µm pores for drinking water samples and paper filters with 8-12 µm pores for all other samples. However, on the one hand paper filters are used to retain metal cations (Engin et al., 2010), with at least part of ionic exchanges (Weltje et al., 2003; Frew and Pickering, 1970) which suggest that these filters have a negative charge that does not promote the retention of negatively charged particles, such as viruses and bacteria, at pHs generally observed in the environment (Michen and Graule, 2010; Harden and Harris, 1953). On the other hand polyamide filters may have a negative charge, but it appears to be much weaker than that of cellulose-based filters (Weltje et al., 2003). To our knowledge, the literature does not deal with electrical interactions between anions and the polyamide membrane, suggesting that we can neglect the adsorption of viruses to the surface of these membranes (moreover and to our knowledge, these materials seem to have been never proposed to immobilize viruses from water in a first concentration step). Thus, one can assume that the pre-filtration did not bias virus enumeration. Our RT-qPCR measurements may have suffered from inhibitions. Lodder et al. (2010) working on surface water for the production of drinking water found, with RT-qPCR measurements, 88% and 40% of samples negative for noroviruses using water undiluted and diluted 10 times, respectively; and the same for rotaviruses with 94% and 70% negative samples, respectively. Many authors suggest

diluting environmental samples before performing RT-qPCRs (Widjoatmodjo et al., 1992; Monteiro et al., 1997; Eckhart et al., 2000; Scipioni et al., 2008), even though these dilutions increase the detection thresholds (Schrader et al., 2012). Inhibitions are generally the result of organic compounds (Pontiroli et al., 2011; Demeke and Jenkins, 2010; Wei et al., 2008; Peist et al., 2001; Burkardt, 2000; Powell et al., 1994; Richards 1999; Lantz et al., 1997; Monteiro et al., 1997; Katcher and Schwartz, 1994; Weyant et al., 1990), more abundant in wastewater (Da Silva et al., 2007; Toze et al., 1999) or in rivers downstream of wastewater discharges (Hata et al., 2014). Conversely, inhibitions seem absent for authors who have worked on groundwater (Haramoto, 2018) and drinking water (Haramoto et al., 2004). *A priori* in our study, the waters with the highest content in organic compounds include the leachate from the *Doña Juana* landfill and the irrigation water from *La Ramada*. Several strategies can be considered to remove inhibitions (Tesson et al., 2019; Schrader et al., 2012); we used two of these strategies: for environmental samples the precipitation of viruses with polyethylene glycol (Demeke and Adams, 1992) before starting RNA extraction (Fang et al., 1992; Wilkins and Smart, 1996), and for faecal samples the addition of chloroform during the extraction of viral RNA (Chaturvedi et al., 2008) and the use of mixtures 'phenol+chloroform+isoamyl alcohol' (Wiedbrauk et al., 1995), although Pachner and Delaney (1993) showed that extraction with a 'phenol+chloroform' mixture was not sufficient to completely remove PCR inhibitors. More specifically for noroviruses, the confrontation between positive samples for RT-qPCR measurements and mouse intestinal villi infections suggests higher detection thresholds and/or sometimes inhibitions in RT-qPCR (cf. section II.2.).

Knowing that the contamination of water results from the discharge of pathogens with the stools of infected persons, it indirectly reflects the state of health of the populations as already suggested for AGEs by Tesson et al. (2019) or more recently for Covid-19 by Rimoldi et al. (2020). But, since AGEs are frequent and generally mild, people rarely try to identify the pathogens causing them, this detection being generally restricted to the most serious cases, e.g. cases leading to hospitalization. Access to the aetiology of only these cases gives a distorted picture of the population health. In Colombia, a recent study of 431 cases of AGEs in children <5 years in Bucaramanga gave by decreasing frequency first viral aetiologies (norovirus GII (17% of cases), rotavirus (9%), sapovirus (6%), astrovirus (4%), norovirus GI (3%), adenovirus (3%)), second bacterial aetiologies (enteroaggregative *E. coli* (14%), enteropathogenic *E. coli* (7%), enterotoxigenic *E. coli* (4%)), *Campylobacter* spp. (3%), *Salmonella* spp. (3%)), and third unicellular parasite-type aetiologies (*Blastocystis hominis* (10%), *Entamoeba histolytica* (7%), *Giardia duodenalis* (6%), *Cryptosporidium* spp. (3%))

(Farfán-García et al., 2020), showing the diversity of aetiologies causing AGEs and the preponderance of viral aetiologies (around 42% of cases). Note that *Shigella* spp. explained only 0.2% of AGE cases (Farfán-García et al., 2020).

Contamination of chard: Chards were used as a model of a leafy-vegetable that can be eaten raw. Their contamination, monitored by *E. coli* as an indicator of faecal contamination, was important during their production when using heavily contaminated water for irrigation, as was the case in *La Ramada*; in contrast, contamination during production was low or negligible near *Cota* where chards were irrigated with groundwater, and near *Muchuelo Alto* where surface water far from wastewater discharge areas. But chard contamination could evolve strongly in the post-harvest stages until the final purchase by the consumer: on the one hand, the washing carried out at *La Ramada* before the departure of harvested products could increase contamination, while the washing carried out with drinking water in the wholesale market of *Bogotá (Central de Abastos)* decreases faecal contamination (*E. coli* removals were at least 1 to 2 log₁₀, or even more); on the other hand, the contamination of chard increased over time on the stalls of nearby merchants, caused certainly by handling of vegetables with dirty hands. These results are consistent with current knowledge, however, the contamination of chards during their production could be higher than that suggested in other studies (Hall et al., 2012), even if Holvoet et al. (2014) noted the presence of faecal indicators in more than 59% of samples of water used in lettuce irrigation in West Flanders (Belgium) and Kokkinos et al. (2012) noted an increased risk of leafy-vegetable contamination by human enteric viruses during their production in three European countries (Greece, Serbia and Poland). However, studies relating to the contamination of leafy vegetables eaten raw are limited (European Food Safety Authority, 2014), especially during production (Renault et al., 2017), with a distinction between external contamination and internal contamination following internalization of pathogens, especially viruses internalized mainly *via* the roots (DiCaprio et al., 2015; DiCaprio et al., 2012) but also bacteria passing from the surface of leaves into the phyllosphere (Hartmann et al., 2017). Studies have highlighted the impact of handling and have suggested that unhygienic post-harvest handling was the main source of contamination of leafy vegetables, and even other crop productions (Pérez-Rodríguez et al., 2019; Hall et al., 2012; Ensink and van der Hoek, 2006). Treatments for freshly cut leafy vegetables are limited (Renault et al., 1994); washings often allow a removal of about 1 log₁₀ or less (Uhlig et al., 2017; WHO, 2006), but they can also promote cross-contamination (Murray et al., 2018), and other treatments such as cold and storage in a modified atmosphere packaging can promote

the maintenance of pathogenic viruses in humans (Baert et al., 2009).

In contrast to *E. coli*, we detected only norovirus GI on the surface of chard during their production among all the pathogens monitored in RT-qPCR and qPCR (norovirus GI, norovirus GII, *Shigella* spp., *Salmonella* spp.), and this only on the site of *La Ramada* (*Mosquera*); no pathogens were detected at the other sites. Using different methods (RT-qPCR, immuno-dot-blot, culture on mouse intestinal villi), we obtained positive chard samples at different stages between production at *La Ramada* and nearby stores for norovirus GI, but also for norovirus GII, confirming the possibility of contamination after production. In addition, some samples negative in RT-qPCR were found to be positive by immunodot-blot and after culture on the mouse intestinal villi, in particular a sample of chard sold on the wholesale market of *Bogotá* (*Central de Abastos*), suggesting that RT-qPCR did not allow us to see everything (problem of detection threshold and/or inhibition?) and that at least some of the viruses on the surface of chard were still infectious (cf. **Chapter III** of this manuscript). Thus, the faecal contamination of the irrigation water of *La Ramada* resulting from the discharges of wastewater from *Funza* leads to the presence of human enteric pathogens in this water and on the leafy-vegetables irrigated by this water. Although we have too little data to address the prevalence of leafy-vegetable contaminations, a simple average gives a prevalence of 33% for norovirus contamination during production.

An older study carried out in parallel in Belgium, Canada and France (but more credible especially in Canada due to the high number of analyses carried out in this country) resulted in prevalence for leafy vegetables of 33% (2/6), 28% (181/641) and 50% (3/6), respectively, with a higher frequency of contamination by norovirus GI than by norovirus GII (Baert et al., 2011). The ideal would be to work on the prevalence of samples representative of a pool of consumers to be supplied, as did Loutreul et al. (2014) for France; the authors of this study found prevalence of around 12% and less than 1% for noroviruses GI and GII, respectively, on different types of green salads (chicory, lettuce, lamb's lettuce ...) (Loutreul et al., 2014). For the region addressed in this PhD, we would have to take into account the sources of supply in *Bogotá* and the surrounding towns, production methods and distribution channels (Eitzinger et al., 2014; Guarín, 2013; Montoya and Mondragón, 2010). Note that, like Loutreul et al. (2014), we found a higher prevalence for GI noroviruses than for GII noroviruses; environmental transmission of GI norovirus is sometimes considered to be greater than that of GII norovirus (Cook et al., 2019), but other studies have found a non-negligible prevalence of norovirus GII (in Canada in Baert et al. (2011)), or even a much higher prevalence of norovirus GII than that of noroviruses GI (Gao et al., 2019). The other

pathogens that were not detected on chard in our PhD work are often mentioned as contaminating vegetables: *Salmonella* spp. on Swiss chard (Hartmann et al., 2017), *Shigella* spp. on and in leafy greens (Jo et al., 2019).

Beyond the existence or not of correlations between concentrations of faecal contamination indicators and pathogens already widely discussed in the literature (see **Chapter II**), there is the problem of the similarity of their behaviour from the production of leafy-vegetables to their final purchase by consumers. In particular, the removal of *E. coli* caused by the washing of chard at the wholesale market in Bogotá (*Central de Abastos*) and indirectly observed in this study, was it transposable to human pathogens? We can suggest responses varying with pathogens and linked to the interactions between the pathogen and the external surface of leafy-vegetables, these interactions being highly dependent on the air moisture: the adsorption of pathogens to the external surface of the plants brings into play different forces – including Van der Waals forces, dielectric forces, hydrophobic interactions also called Lewis acid-base forces, and Born forces (Van Oss, 2006) – in a very special at least three-phase environment (air, water which can be strongly linked to the pathogen and to the plant surface, and solid (pathogen and plant)), or even four-phase environment, distinguishing the pathogen and the plant by their potentially very different surface properties.

Air contamination: *E. coli* and T.C.\E. coli were detected in about one quarter and one half of the air samples, respectively. Surprisingly, *Shigella* spp. was detected in two thirds of the samples while *Salmonella* spp. has never been detected and noroviruses have been detected in only a few samples (we did not seek to detect rotaviruses and *Cryptosporidium* spp. on these samples). The percentages of samples positive for *E. coli* and for T.C.\E. coli were slightly higher in the rainy season. Taking into account the direction of the wind, its intensity and the air temperature when sampling air, as well as the rains during the days before sampling air, we did not show a significant contribution of the *Doña Juana* landfill to the presence of these microorganisms in the air. However, the concentrations of *E. coli*, T.C.\E. coli and *Shigella* spp. seemed affected by the direction of the wind and by the rains during the days preceding the measurements. It was as if the rains kept the microorganisms on the soil. Moreover, the concentrations of T.C.\E. coli appeared to be higher for westerly winds suggesting that coliforms other than *E. coli* could easily originate from rural environments, in accordance with their ability to live and multiply in the environment. The impact of wind direction on *E. coli* and *Shigella* spp. appeared less clear, with perhaps higher concentrations of *E. coli* and *Shigella* spp. for north and south winds, respectively. *A priori*, we can think that

E. coli are emitted mainly from excrements of human and animal origins released into the environment. We can thus imagine that certain zones are hot-spots for emissions of *E. coli*, in particular those where wastewater is treated and/or discharged (e.g. the *Salitre* wastewater treatment plant, the *Bogotá* river and the downstream parts of the *Tunjuelito* and *Fucha* rivers), the *Doña Juana* landfill receiving soiled adult and children diapers and toilet paper from the city of *Bogotá* which are scattered on the soil before being covered within 24 h (UAESP, 2020) and some animal husbandry areas (Hansen et al., 2020; Yu et al., 2020; Rafique et al., 2020). The concentrations of *Shigella* spp. in the air were sometimes surprisingly high. One could imagine that *Shigella* spp. come from a wide variety of sources, including plants that this pathogen can infect (Jo et al., 2019), and the waste contained in garbage trucks that arrive at the landfill (Madsen et al., 2016). The higher percentage of samples positive for *E. coli* and for T.C.\E. coli during high-rainfall months could be explained by a better resistance of these microorganisms at higher relative air humidity (Krammer and Assadian, 2014). Regarding more specifically *Shigella* spp., it should be recalled that the qPCR measurements were taken after sampling from media which were previously incubated for 24 h to enumerate *E. coli* and T.C.\E. coli; we cannot therefore exclude a growth of these pathogens and the analyses made on the concentrations should perhaps, as a precaution, only be carried out on the positive or negative character of the samples. All the more so since *Shigella* spp. does not appear to be strongly involved in the aetiology of AGEs in this region (Farfán-García et al., 2020). In fact, some authors had done so to better distinguish positive and negative samples in contexts of low concentrations of *Shigella* spp. (Zhou et al., 2015; Ishii et al., 2014), and several authors noted significant exponential growth under aerobic conditions, at close temperature (30-37°C) with culture media that were however different (close pH) for *Shigella* spp. (Hentges, 1969; Hentges, 1967) or *Salmonella* spp. (Gehring et al., 2012).

II.2. Detection of total noroviruses and infectious noroviruses

In view of the importance of noroviruses in the aetiology of AGE (Dennehy, 2019; Hoffman et al., 2015), it seemed useful to develop a method of culture of these viruses in order to be able to pass from genomic copies (GC) enumerations to the enumerations or, at least, the detections of infectious viruses; a criticism of microbial risk analyses (QMRA) based on genome copy concentrations is that the latter could be much greater than the infectious virus concentrations (Simhan et al., 2020). Some alternatives have been proposed to conventional

measurements of genomic copies, in particular those making it possible to indirectly assess the integrity of the capsid by pre-treatment of the particles with monoazides (EMA, PMA, PMA_{xx}) (Prevost et al., 2016) with recent improvements by Lee et al. (2018), but these methods remain indirect and limited to particular contexts. Recall that several norovirus culture trials have been carried out with mixed success (Oka et al., 2018; Ettayebi et al., 2016; Jones et al., 2015; Karst et al., 2014; Taube et al., 2013 ; Straub et al., 2011; Lay et al., 2010; Duizer et al., 2004; Wobus et al., 2004).

Our norovirus culture proposal is original because it uses isolated mouse intestinal villi as a cell model for wild-type norovirus. Until now, there was no cell model that readily allowed cultivation of human norovirus. Primary villus culture had previously been used for rotavirus type A replication (Guerrero et al., 2010). It made it possible to cultivate human noroviruses, suggesting on the one hand that there are in this cell model all the receptors required by human noroviruses or that the latter share certain receptors with mouse noroviruses, on the other hand that this cell model has all the biochemical machinery for virus replication. This cell model allowed the replication of human norovirus for most samples that were positive for this virus by other detection techniques (36/40). This suggests a high sensitivity of the system. Thanks to this cellular model, it should thus be possible to study important aspects, not yet resolved, such as cell receptors, the mechanisms of entry of the virus into the cell and the biochemistry of the infectious process (Bhar and Jones, 2019; Ettayebi et al., 2016; Marionneau et al., 2002; Dolin et al., 1975). Likewise, this model should be useful for studying drugs that inhibit the infectious process. Likewise, in this model, we corroborate that it is necessary to activate norovirus with trypsin as suggested by Jones et al. (2015) and Oka et al. (2018) to cut the structural protein VP1 and give the virus the ability to access cellular receptors, possibly because domains necessary for this purpose become more exposed (Mallory et al., 2019; Hutson et al., 2004; Hardy et al., 1995); this use makes it possible to mimic *in vivo* conditions in accordance with the current understanding of norovirus binding and replication sites in humans (Bhar and Jones, 2019; Ettayebi et al., 2016; Marionneau et al., 2002; Dolin et al., 1975).

Among the 54 environmental samples (air, water, chard) considered for culturing on mouse intestinal villi, 40 were retained following positive tests for the presence of norovirus by RT-qPCR and/or by immunodot-blot, the other 14 samples being negative for these two preliminary tests. Of the 40 samples selected, 4 were positive for both methods (RT-qPCR and immunodot-blot), 26 were positive by immunodot-blot only, and 10 were positive by RT-qPCR only. 36 samples that were RT-qPCR and/or immunodot-blot positive for the presence

of human norovirus were positive for the presence of infectious virus: 85% (22/26), 100% (10/10) and 100% (4/4) of samples where viruses were detected respectively by immunodot-blot only, by RT-qPCR only, and simultaneously by immunodot-blot and RT-qPCR, respectively. This result seems all the more interesting as the detection thresholds for the concentrations of infectious virus are within the ranges proposed for the detection thresholds in RT-qPCR: assuming that an infectious virus is sufficient to infect a mouse intestinal villus, the detection thresholds of infectious virus concentrations would be around 700 infectious viruses per gram of excrement (vi.g^{-1} excrement), 15 vi.L^{-1} water, 0.2 vi.g^{-1} chard and 7 vi.m^{-3} air, against $300\text{-}1200 \text{ GC.g}^{-1}$ excrement, $15\text{-}50 \text{ GC.L}^{-1}$ water, $0.3\text{-}0.7 \text{ GC.g}^{-1}$ chard and $5\text{-}15 \text{ GC.m}^{-3}$ air. Thus, one can think that most of the viruses detected by RT-qPCR were infectious. However, we observed that some isolates took longer time to lyse cells (12h-24h), probably because of the reduced number of virions present. We did not have access to detection thresholds for the immunodot-blot, but we can hypothesize detection thresholds in the same ranges, given the results that were positive sometimes only in RT-qPCR, and sometimes only in immunodot-blot. The fact that we often detected noroviruses by one method but not by both methods simultaneously could suggest that the detection thresholds of these methods were of the same order of magnitude and that concentrations were close to the detection thresholds of these methods. We could then imagine that details sometimes make the detection better in RT-qPCR (for example if the immunodot-blot measurement is altered by excessive washing of the membrane after the addition of the antibodies (Stuart and Greenstone, 1990)), sometimes better in immunodot-blotting (for example when RT-qPCR is inhibited (Pontiroli et al., 2011)).

Curiously, the 4 samples where the viruses detected by immunodot-blot only were not cultivable were water samples where it could have been assumed that the viruses retained their infectiousness longer than in air or when they are adsorbed on plant outer surface and exposed to more or less dry air (Figure 1; Table 2 of Chapter III).

Similarly, out of the 37 human stool samples analysed from people with clinical symptoms of AGE, only 2 samples were positive simultaneously by immunodot-blot and RT-qPCR for the detection of human norovirus, and 3 samples were only positive in immunodot-blotting. The other 32 samples were negative for both methods; It should be noted that 11 stools were positive by immunodot-blot for rotaviruses, of which 3 were also positive for noroviruses (the stools of the 3 elderly people were positive for rotaviruses). Positive stools were from children <5 years old only.

Shigella spp. was, among the other pathogens, the most frequently pathogen detected in

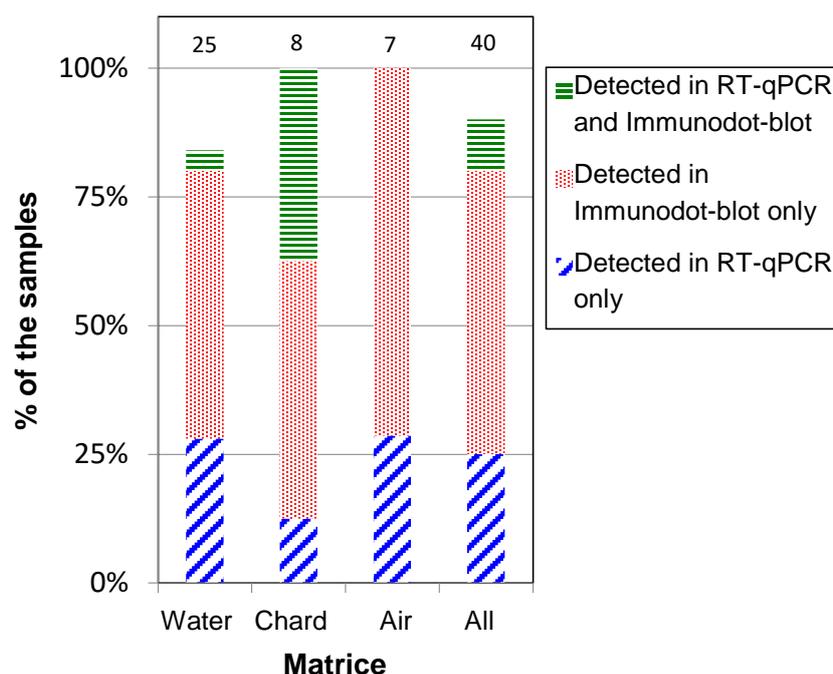


Figure 1: Percentages of environmental samples (water, chard, air) positive for the presence of human norovirus in immunodot-blott and/or in RT-qPCR, with noroviruses cultivable on the mouse intestinal villi. (Above the histogram are the total numbers of samples tested for each type of medium studied).

the stool, with 17 children <5 years and the 3 elderly patients positive, in contrast to the results of Farfán-García et al. (2020). *Shigella* spp. appeared 5 times in combination with viruses: 2 times with both rotavirus type A and norovirus, and 3 times with rotavirus type A only. Only one sample was positive for *Cryptosporidium* spp., corresponding to a low prevalence as in Farfán-García et al. (2020).

II.3. Health risks for the population of Bogotá and its surroundings

If the work carried out does not give us all the elements necessary for the implementation of a quantitative assessment of microbial risks (Membré and Guillou, 2016), it nevertheless allows us to put forward some elements of reflection.

Surveys of 133 homes in the neighbourhoods of *Usme* resulted in an estimate of approximately 22% of the population with AGE in the year preceding the surveys. This value is lower than the orders of magnitude proposed for France (33% according to Van Cauteren et

al. (2012)) and for the USA (55% according to Lively et al. (2018)), but it must be taken with caution for 2 reasons: on the one hand the very limited number of surveys and their carrying out over a limited period with its peculiarities, on the other hand the restricted definition of AGE in this work focusing on diarrhoea and leading to ignore cases of vomiting without diarrhoea which may not be negligible (see for example the work of Tesson et al. (2019)). As in Colombia, the notification of AGE is still based on diarrhea, we recommend add to the notification process of AGE in Colombia vomiting events without diarrhea; in this way, more complete information will be obtained on the epidemiology of AGE.

A priori, the quality of tap water does not seem to be an important risk factor even if we noted the presence of infectious human norovirus in one sample of tap water in a school (see **Chapter III**). Conversely, the contamination of market gardening products eaten raw seems to be real on certain production sites, *La Ramada* in particular where surface water used for irrigation is highly contaminated by discharges of untreated wastewater or wastewater subject to a primary treatment only. In view of *E. coli* removal, outer surface contamination of vegetable crops seems to be partially or totally eliminated by washing them with drinking water at the wholesale market in *Bogotá (Central de Abastos)*, provided that this washing has at least the same impact in terms of removal on human enteric pathogens as on *E. coli*. However, it cannot be excluded that internalized contamination (especially virus internalization) exists and then cannot be removed by washing. With the short-term development of wastewater management in *Bogotá* and in the surrounding towns (see **Chapter III**), it is reasonable to assume that contamination during production will decrease quite quickly, or even disappear. However, it is important not to fall behind in the extension of the *Salitre* wastewater treatment plant and to start the construction of *Canoas* new wastewater treatment plant as soon as possible. But these constructions and these developments will perhaps not be enough: the wastewater discharged into the *Bogotá* River or its tributaries without treatment or after a primary treatment by towns in the vicinity of *Bogotá* participate in the contamination of the water used for irrigation, and it is important that wastewater treatment be generalized to these municipalities with at least secondary treatment. This should allow at least a removal in human enteric pathogens of 2 log₁₀ during the wastewater treatment which, combined with other actions (waiting time between last irrigation and harvest, post-harvest washing, etc.), would make it possible to be in the frame of mind of the guidelines laid down by the WHO (2006). Moreover, we recommend strengthening the sanitary control of vegetables that can be consumed raw in Colombia, from their production to their purchase by end customers, and to consider their packaging to avoid

their contamination during handling.

However, post-harvest handling, particularly on the stalls of nearby stores, leads to new contamination of the products although less than that due to irrigations with contaminated water; this observation has already been made in other places (Pérez-Rodríguez et al., 2019; Hall et al., 2012; Ensink and van der Hoek, 2006) and can only be resolved through better personal hygiene and/or changes in distribution channels aimed at limiting product handling to a strict minimum. Our work seems to show stricter food hygiene practices in homes with elderly people than in other homes (see **Chapter V**).

An unexpected result of this work is certainly the high frequency of contamination of the air with *Shigella* spp. (cf. **Chapter IV**) and, perhaps, the link to be made between this high frequency and the high frequency of presence of this pathogen in the stools of people with acute gastroenteritis in our study (20/37), this pathogen being the only one detected in more than half of the cases where it was present (cf. **Chapter III**), although *Shigella* spp. explained very few acute gastroenteritis in *Bucaramanga* northeast of *Bogotá* in Colombia (Farfán-García et al., 2020). The *Doña Juana* landfill does not seem to be a privileged place for aerosolizing this pathogen which, until now, was only known to infect humans and other primates (McLennan et al., 2018). Its recently observed ability to infect plants and in particular fresh vegetables (lettuce, tomatoes and green peppers) (Jo et al., 2019) allows us to consider aerosolization from more diffuse sources.

III. Perspectives

In the short term, some additions must be made to our work to facilitate its scientific valuation, or even an applied valuation:

- additional analyses on the surveys carried out in 133 households, including the analysis of the answers to questions on health practices (in the face of AGE cases) and, if possible, an in-depth study of the correlations between variables that may hide cause and effect relationships between certain practices and AGE cases;
- a new analysis of data on contamination by *Shigella* spp. of air by working only on their presence or absence rather than on their concentrations due to our protocols which may have changed the latter (see subsection **II.1.**).

In the medium term, several advances/new developments can be considered in different

areas:

- Culture of human norovirus on isolated mouse intestinal villi: it currently allows us to have a very sensitive test for the presence/absence of infectious norovirus in environmental and faecal samples, but it does not allow us to enumerate them. Two alternative avenues could be considered for moving to the enumeration of infectious viruses:
 - (i) the inclusion of the current culture method in a protocol suitable for a MPN (Most Probable Number) analysis. However, a single sample to be characterized would then require several cultures to be carried out in parallel (several dilutions and several replicates per dilution) generating high costs in terms of required sample amount, of animal material, and of laboratory working time;
 - (ii) the identification of the types of cells actually infected with noroviruses within the mouse intestinal villi and an attempt to work on an isolated cell strain, with probably the need to find cancerous lines of the cells in question.

Beyond that but a little off topic for us, medical virology specialists might be interested in using the mouse intestinal villus model to address unresolved questions in the medical field such as the cell receptors involved in the adhesion of the virus to cells, the mechanisms of entry of the virus into the cell, the biochemistry of the infectious process;

- Contamination of leafy vegetables eaten raw from production to final purchase: our results are mainly based on indicators of faecal contamination (*E. coli* and T.C./*E. coli*) without monitoring performed on only one or a few batches well identified between production and final purchase. A future work could be dedicated to a sector-type approach by following one or a few batches of leafy-vegetables produced on one or two plots, for example cultivated in periods requiring little or a lot of irrigation, and by improving our experimental protocols to better count some human enteric pathogens (for example norovirus GI, *Shigella* spp. and *Cryptosporidium* spp.);
- Participation of the Doña Juana landfill in the contamination of the atmosphere with human enteric pathogens: our work did not allow showing a contribution of this landfill to the contamination of the atmosphere of Bogotá and its surroundings, and the treated leachate from the landfill contained few pathogens (noroviruses never detected, *Shigella* spp. detected sometimes). But our work does not prove that pathogen aerosolization from the landfill could be neglected. One could imagine experiments devoted to the quantification and description of landfill impacts on the environment. Concerning more specifically air quality, we may imagine combining the monitoring of climate and air

- contamination at different distances from the landfill, the monitoring of uncovered waste surfaces within the landfill and of pathogens in the waste, and the possibility of aerosolizing waste and/or pathogens from waste as function of waste properties (moisture, temperature ...) and of climate (wind speed and direction ...);
- Contamination of surface water: it would be interesting to have an integrated approach to the determinants of surface water quality by coupling all water flows (rain, runoff, rivers, canals, pumping stations, wastewater, human withdrawals ...) to faecal contamination indicator flows and pathogen flows in order to better understand the behaviour of complex environments such as *La Ramada* where the direction of water flows can change with the seasons.

In the long term and in the context of *Bogotá* and its surroundings, it would be useful to combine in a systemic approach (i) the fate of human pathogens in the environment, (ii) individual food, hygiene and health practices (with the factors that may affect them, such as regulations and communication), and (iii) population epidemiology (combined if possible with a health economics approach). This type of systems approach could lead probably to very relevant quantitative microbial risk assessments (QMRA) and allow the evaluation of different scenarios of water, waste and agriculture management. However, this type of approach would require bringing together people with highly complementary skills.

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