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Diagnosis and genetic characteristics of potential pathogens in children under five years of age with diarrhea

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1. INTRODUCTION

Acute diarrhea is a major public health issue, being a leading cause of mortality and morbidity, particularly in developing countries [1]. More than half a million deaths of children under the age of five worldwide are caused by diarrheal diseases each year. Countries with a lower socio-demographic index (SDI) are disproportionally affected [2]. Overall, it is the second most frequent cause of death in children between one month and five years of age [3]. The new Sustainable Development Goals (SDGs) aim to reduce the mortality rate of children under five years of age to <25 deaths per thousand live births by 2030 [4]. To achieve this goal, it is extremely important to diagnose, treat and prevent diarrheal diseases.

A number of studies have reported estimates of the burden caused by diarrheal diseases, but further studies are urgently required to fill current information gaps and inform intervention strategies at the local level. Epidemiological data on potential pathogenic agents, sources of environmental contamination, animal reservoirs as well as on genetic characterisation are needed to identify populations at risk, implement preventive measures and guide drug development.

So far, in Gabon, a Central African country, there is no comprehensive data on the aetiology of diarrhea in paediatric patients. Researchers in this country have focused on either bacterial [5], viral [6] or specific parasitic causes of diarrhea [7–9].

Moreover, diarrhea is still causing an estimated 28.6 deaths per 100,000 population and has a considerable impact on disability adjusted life years (DALYs) in Gabon in 2015 [10]. Therefore, it is time to pay more attention to the collection of evidence-based data on the prevalence, diversity, or genetic variability of pathogens present in children with diarrhea, as well as diagnostic tools.

1.1. Diarrhea

1.1.1. Defining Diarrhea and diarrheal disease

A common definition of diarrhea is the passage of at least three loose or watery stools per day or at an unusually high frequency for the individual due to an abnormal increase in daily stool fluidity, frequency and volume from what is considered normal for an individual [11]. It is a common symptom of a gastrointestinal infection. Diarrheal diseases are a collection of diseases caused by multiple viral, bacterial, and parasitic organisms that share the common symptom of diarrhea. Diarrheal diseases are considered acute when they last for less than 14 days [12].

1.1.2. Epidemiology

An estimated 2.5 billion cases of diarrhea have occurred among children under five per year over the past two decades [11]. Diarrheal diseases are responsible for one in nine child deaths worldwide, making them the second leading cause of death in children below five [13,14]. In 2016, diarrhea was the eighth-leading cause of mortality responsible for more than 1.6 million deaths. Among these, more than a quarter occurred among children below five and most of the fatal cases (about 90 %) occurred in Asia and sub-Saharan Africa [15]. Although diarrhea is globally present among all regions, evidence shows that it disproportionately affects developing nations with poor access to health care, safe water and proper sanitation [2].

1.1.3. Pathophysiology of acute diarrhea

Diarrhea results from the disruption of the entero-systemic water cycle, through disruption of the processes of absorption and/or secretion of electrolytes, primarily sodium. One of the main mechanisms leading to diarrhea is that certain product (e.g.: cholera enterotoxin, and certain viral toxins, such as NSP4 of rotavirus) stimulate diverse signalling pathways that increase the secretion of sodium and chlorine into the intestinal lumen. This generates water and causes watery diarrhea. The absorption-related mechanism leads to invasive diarrhea characterised by enterocyte destruction and villous disruption [16,17].

1.1.4. Infectious agents responsible of diarrhea

The frequency of pathogens causing diarrhea may vary depending on the geographic location. In principle, more than one pathogen may be implicated in an episode of diarrhea. Several microorganisms (parasites, bacteria and viruses) are frequently associated with diarrhea. Among these, common infective agents include enteric adenovirus (serotypes 40 and 41), *Aeromaons* spp., *Entamoeba histolytica* (amoebiasis), *Cryptosporidium* spp., typical enteropathogenic *Escherichia coli* (t EPEC), enterotoxigenic *E. coli* (ETEC; both ST and LT), norovirus (NoV), non-typhoidal *Salmonella* spp., rotavirus A (RVA), *Shigella* spp., *Vibrio cholerae* and *Clostridium difficile* [2].

These pathogens are spread by the faecal-oral route either directly from person to person or indirectly via the consumption of contaminated food or water.

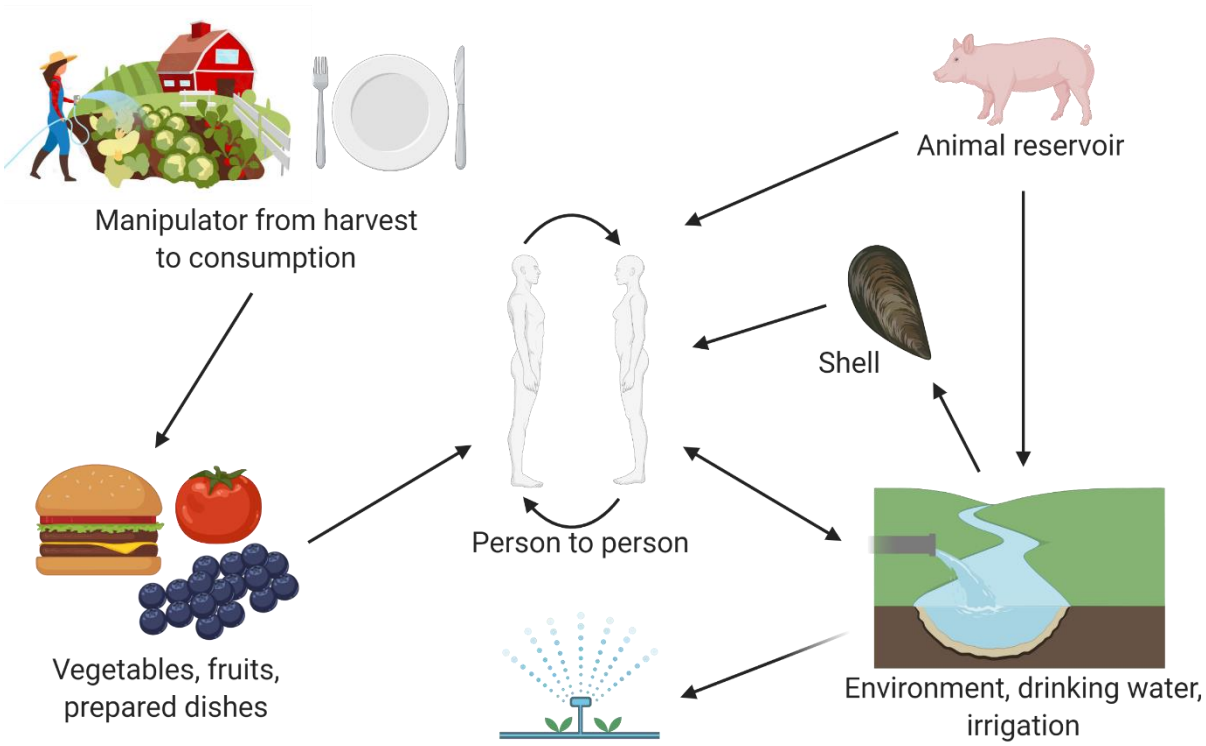

Figure1: Routes of transmission of enteric pathogens.

Source : Bouseettine *et al*., Emerging and Reemerging Viral Pathogens. 2020 [18] (with permission provided by Elsevier and Copyright Clearance Center)

In high income countries, viruses are more frequently found among gastrointestinal infections than in low- and middle-income countries, where the frequency of bacterial and protozoal agents is also important. This is in accordance with the study conducted by Kotloff et *al*. 2013 showing that rotavirus, *Cryptosporidium*, ETEC and *Shigella* spp. were strongly associated with moderate to severe diarrhea. Among all enteropathogens, RVA is widely recognized as the leading cause of moderate to severe diarrhea in children [1]. An estimated 128,500 rotavirus deaths worldwide, with an estimated 104,733 deaths in sub-Saharan Africa among children aged 0-59 months [19]. Therefore, RVA vaccines have become an international priority for reducing the mortality associated with this infection [20]. The two main vaccines Rotarix (GlaxoSmithKline Biologicals Rixensart, Belgium) and RotaTeq (Merck and Co., Inc. Whitehouse Station, New Jersey, USA) are effective in reducing mortality and morbidity associated with RVA infection [21,22]. Although the vaccines have been approved, their effectiveness appears to be significantly reduced in low-income countries [23,24] and program implementation has not yet reached many countries, particularly in Africa and Asia.

In developing countries, *Cryptosporidium* is known to cause prolonged and persistent diarrhea, malnutrition and growth retardation in immunocompetent children and severe diarrhea in immunocompromised people [25]. Even in an environment with modern diagnostic facilities, such as in the United States of America, it is estimated that only about 1% of cases are diagnosed and reported [26]. As a result, in Africa where (i) routine faecal microscopy is not available particularly for *Cryptosporidium*, (ii) advanced diagnostic tools such as immunofluorescence and polymerase chain reaction (PCR) tests are not widely available and (iii) the awareness and knowledge of health professionals is insufficient, cryptosporidiosis is severely underestimated and under-diagnosed, and preventive measures are lacking.

1.1.5. Treatment

The management of acute diarrhea always begins with oral rehydration by using the oral rehydration salts. In some cases, additional interventions may be

required depending on the clinical manifestations and/or the identification of the potential pathogens causing diarrhea. [27].

1.1.6. Prevention

Diarrhea can be prevented by improving life conditions (access to safe drinking-water, health education about how infections spread, good food hygiene), breastfeding during the first six months of life and vaccination against Rotavirus [28].

1.2. *Cryptosporidium*: an overview

1.2.1. Taxonomy and biology of *Cryptosporidium*

Cryptosporidium spp. was first recognized by Tyzzer in 1907, who described *Cryptosporidium muris* in the stomach of laboratory mice [29,30]. Shortly after, the taxonomic status of the genus *Cryptosporidium* became clear. Genus *Cryptosporidium* (family *Cryptosporidiidae)*, traditionally found to be more closely related to the gregarines, is a member of the phylum *Apicomplexa* [31]. These parasites, like enteric coccidia, are monoxenic (single host) parasites in the digestive and/or respiratory tracts of vertebrate hosts. *Cryptosporidium* spp. develop within the brush-like border of the host epithelial cells (and not in the host cell itself) [32–34]. The parasite has a complex life cycle comprising sexual and asexual stages. There are three stages of development: merontes, gamontes, and oocysts. The endogenous developmental stages take the form of small basophilic bodies (3-6µm) attached to the luminal surface of the host epithelial cells, whereas the exogenous oocysts take the form of bright-phase ovoid bodies (5-7x4-6µm) containing four sporozoites and an eccentric residual body [35].

1.2.2. Epidemiology and transmission

Currently, there are more than 30 species that have been included in the genus *Cryptosporidium*, with only a few species (*C. hominis, C. parvum, C. meleagridis, C. felis, and C. canis*) commonly found in humans [36–38].

During the last years, the number of cryptosporidiosis cases reported worldwide reached 3 cases per 100,000, although this number is likely underestimated [39].

The prevalence of infection ranged from 0.0% to 86.0% and varied from one population to another and from one country to another [40]. Globally, *Cryptosporidium* infection rates have been reported to be significantly higher in developing countries compared to industrialized countries [41,42]. Outbreaks of cryptosporidiosis have been frequently reported to be associated with drinking water and swimming pool contact in several countries. This pathogen can be transmitted by direct contact with an infected human or animal, and indirectly via the consumption of contaminated food or water [43]. Recent studies indicate that transmission can also be possible by inhalation of aerosolized droplets via respiratory secretions or by coughing [44].

1.2.3. Clinical features and treatment

1.2.3.1. Clinical features

This apicomplexan parasite infects both immunocompetent and immunocompromised persons. In healthy people with an intact immune system, *Cryptosporidium* infection occurs mostly in children below 5 years old [1]. Clinical symptoms include diarrhea, abdominal cramps, vomiting, headache, fatigue, and low-grade fever [45]. Cryptosporidiosis during infancy infection can also lead to growth retardation, particularly in children with no clinical symptoms [46]. In immunocompromised people such as patients with cancer and transplants undergoing immunosuppressive therapy, acquired immunodeficiency syndrome (AIDS) patients, *Cryptosporidium* is associated with life-threatening diarrhea [47]. For instance, individuals with HIV/AIDS are exposed to a chronic, prolonged form of cryptosporidiosis which is difficult to treat and can even lead to death [48].

1.2.3.2. Treatment

Nitazoxanide (NTZ) is the only FDA-approved drug for the treatment of cryptosporidiosis although this drug is not effective for the treatment of *Cryptosporidium* infection in immunodeficient individuals [49]. Moreover, oral or intravenous rehydration is used whenever severe diarrhea is associated with *Cryptosporidium* infection. This practice is the major supportive treatment for immunocompromised patients [50].

1.3. An overview of important enteric viruses

1.3.1. Rotavirus

1.3.1.1. Classification

Discovered in the intestinal tissue of mice in 1963, this virus belongs to the *Reoviridae* family. Rotaviruses have a non-enveloped, multilayered protein capsid containing 11 segments of double stranded RNA, encoding nonstructural (NSP1-NSP6) and structural proteins (VP1, VP4, VP6, and VP7) [51]. Rotaviruses are divided into serogroups, subgroups, serotypes, and genotypes according to differences in the antigenic properties, genetic similarity, and gene constellations. There are eight major rotavirus groups (A-H) based on the VP6 (inner capsid protein), four of which (A, B, C, and H) are human pathogens [52]. Group A rotavirus is the most common and causes the majority of human rotavirus infections.VP4 and VP7 are the major outer capsid surface proteins and represent the main targets of neutralizing antibodies [21]. Therefore, these two surface proteins also determine the RVA serotypes, designated G type (according to VP7) and P type (according to VP4), respectively. VP7 proteins and VP4 (a non-glycosylated, protease-sensitive protein) are dominant in inducing an immune reaction leading to neutralizing antibodies [53].

In 2008, a new classification system based on all 11 genome segments was proposed by Matthijnssens et *al*. [54]. The aim was to designate the complete genetic constellation of rotavirus following the schematic nomenclature: Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, representing the genotypes of, respectively, the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 genes, with x indicating the numbers of the corresponding genotypes.

1.3.1.2. Clinical features and Epidemiology

Rotavirus is mainly transmitted by the faecal-oral route and the incubation period is typically less than 2 days [55]. Infections lead to a spectrum of clinical symptoms such as acute gastroenteritis (AGE) with mild to severe diarrhea, vomiting and various degrees of dehydration [56].

Rotavirus remains among the most common causes of AGE in children worldwide [57],accounting for approximately one-third of all hospital admissions for diarrhea disease. In 2016, it is estimated that 128,500 deaths (95% UI, 104,500-155,600) were due to rotavirus infections among children. Most of the death cases occur in developing countries in sub-Saharan Africa and Asia.

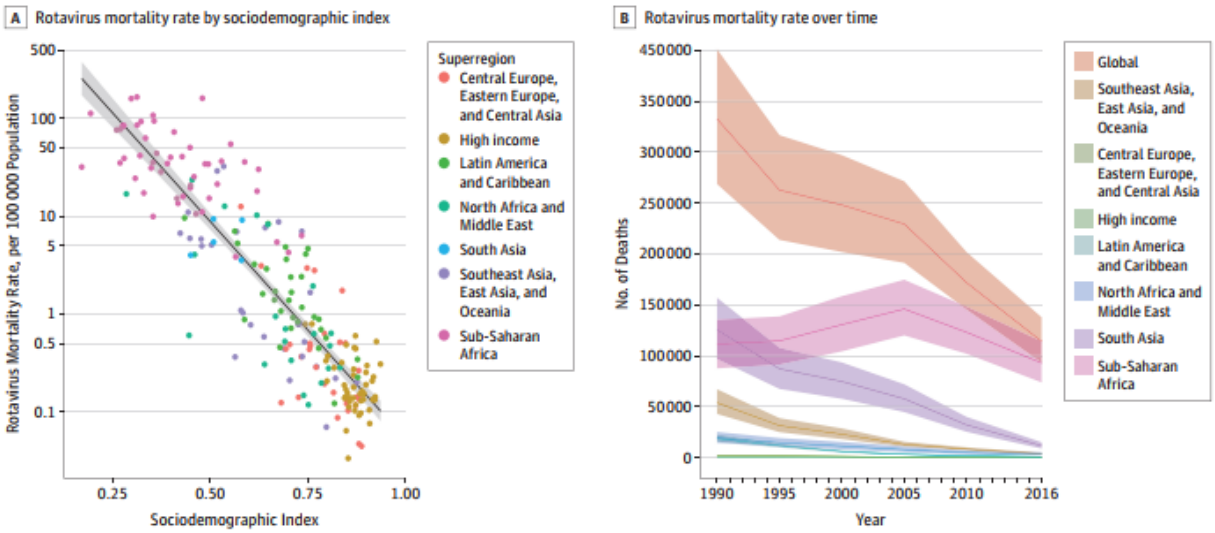

Figure2: Sociodemographic and Spatial-Temporal Patterns in Rotavirus-Associated mortality [58]. A, Rotavirus-associated mortality rate by sociodemographic index in 195 countries, estimated for 2016. B, Rotavirus mortality rate over time, globally and by super region.

Source : Troeger C et *al*., Jama Pediatr. 2018. (with permission provided by CC-BY license: https://jamanetwork.com/pages/cc-by-license-permissions)

A total of 104,733 deaths from rotavirus infection (95% UI, 83,406-128,842) among children under 5 years occurred in sub-Saharan Africa [58].

1.3.1.3. Genetic diversity of rotavirus

Great diversity in circulating rotavirus wild-type strains is observed worldwide. This remarkable diversity in circulating rotavirus strains identified so far, varies over time and across regions [59].

The fluctuations and changes observed for rotavirus circulation are thought to be driven by several mechanisms. These mechanisms include (1) accumulation of

point mutations (genetic drift) that can lead to antigenic changes; (2) gene rearrangement (deletions, duplications and insertions) into coding or noncoding regions, primarily of non-structural genes [60]; (3) genomic reassortment (exchange of RNA segments between strains co-infecting a human host) and potential interspecies reassortments (occurring after direct zoonotic transmission) that are considered to generate new strains and the continued emergence of new strains [61,62].

1.3.1.4. Rotavirus vaccine

Since the World Health Organization has reported that hygienic measures and improved sanitation are not sufficient to significantly reduce the burden of disease due to rotavirus burden, vaccination has been proposed as the optimal strategy. Currently, two vaccines are marketed: Rotarix and RotaTeq [63]. Both vaccines tend to provide partial immunity against moderate to severe disease [64].

Rotarix® (produced by GlaxoSmithKline (GSK)) is a single-strain live, attenuated human G1P[8] vaccine [65].This vaccine was derived from the human rotavirus isolate 89-12 and attenuated by serial passage in cell culture. Rotarix vaccine was licensed for universal vaccination of infants in 2005.This live attenuated human vaccine is produced as “Rotarix®” by GlaxoSmithKline [56].

RotaTeq® (produced by Merck) is a pentavalent, live attenuated vaccine developed using the bovine RVWC3 strain (G6P7[5]) as a genetic backbone for 5 mono-reassortants. This vaccine produced as RotaTeq® by Merck, underwent a successful Phase III clinical trial and was licensed from 2006 onwards in many countries across the world. This human-bovine reassortant vaccine provides clinical protection against five of the most common rotavirus A genotypes, G1, G2, G3, G4 and P[8] [56].

Overall, several studies have reported that both vaccines are highly effective in developed countries with protection rates of 70 to >90% [66–70]. Contrary to developed countries, in some middle and low income countries including South Africa, Kenya, Mali and Vietnam, the efficacy of the vaccines was found to be substantially lower with a protection rate between 20 and 30% [71–73].

1.3.2. Norovirus

Noroviruses (family *Calciviridae,* genus *Norovirus*) are small round structured viruses, non-enveloped with a positive-sense single-stranded RNA genome of around 7.5 kb that consists of three open reading frames (ORFs). ORF1 encodes six non-structural proteins including the viral polymerase. ORF2 and ORF3 encode the major and minor capsid proteins VP1 and VP2, respectively [74,75]. The NoV strains are divided into ten genogroups (G), of which members of genogroups GI, GII, GIV; GVIII and GIX typically cause human gastroenteritis. Phylogenetic analysis has identified at least 48 genotypes that are infectious to humans, with genotype GII.4 being responsible for at least 70% of infections worldwide [76,77].

NoVs are mainly transmitted from food/water or person-to-person via fecal-oral route, affecting adults and children all over the world [78]. Also, vomiting could lead to airborne transmission which has been implicated in some outbreaks [79]. It is estimated that over 200,000 deaths annually, particularly with an important proportion occurring in children from developing countries [80,81].

Clinical features include nausea, abdominal pain, vomiting (mild, self-limited and non-bloody) and diarrhea [79].

Currently, there is no available NoV vaccine, as the necessary line attenuated/ inactivated noroviral particles are not yet being produced. This issue results mainly from the lack of an *in vitro* culture system for these viruses [82].

1.3.3. Astrovirus

Astroviruses (family *Astroviridae*, genus *Astrovirus*) were first described in 1975 by Madeley and Cosgrove in children suffering from vomiting and mild diarrhea. The viral particles were described as non-enveloped icosahedral particles with smooth edges and a distinctive five or six-pointed star structure. Human astroviruses (HAstVs) are divided into two genera: Mamastroviruses (which infect mammals, including humans, cats, and dogs) and Avastroviruses which infect birds [83]. The HAstV genome is around 6.8 kb (range: 6.2- 7.8 kb), positive-sense double-stranded RNA containing ORF1a, ORF1b and ORF2. ORF1a and

ORF1b encode non-structural proteases and polymerase proteins, while ORF2 encodes capsid proteins.

Currently, three groups of HAstV are recognised: classical HAstV (serotypes 1-8), and non-classical HAstV MLB (Melbourne) (MLB 1-3), and HAstV-VA/HMO (Virginia/Human-Mink-Ovine-Like) (VA1-5) [84]. Classic HAstVs are distributed all over the world and are associated with 2 to 9% of cases of acute, non-bacterial diarrheal children, although incidences up to 61% have been reported [85,86].

In general, HAstV infection induces a mild watery diarrhea, associated with vomiting, fever, anorexia, and abdominal pain [87].

1.3.4. Sapovirus

Sapoviruses (previously called “typical human caliciviraídea”) are non-enveloped, positive-sense, single-stranded RNA genome viruses. The sapovirus genome is approximately 7.1 to 7.7kb in size with a poly (A) tail at the 3’-end [88]. The genome contains three ORFs. ORF1 encodes the non-structural proteins, including the RNA dependent RNA polymerase (RdRp), and the major viral capsid protein. ORF2 is predicted to encode the minor structural protein VP2 [89]. An ORF3 has been predicted in some sapovirus strains, but its function is still unknown [88,90]. Overall, fifteen sapovirus genogroups were designated based on the genetic differences of the VP1 encoding sequences, and four of these (GI, GII, GIV and GV) are known to infect humans. Of these, there are 19 sapovirus genotypes comprising seven and eight genotypes for GI and GII respectively, and one genotype for GIV and GV can be divided into two [91]. Human sapovirus infections are associated with AGE, in both sporadic cases and outbreak settings throughout the world and among all age groups [92]. The spectrum of human sapovirus symptoms include diarrhea, vomiting, nausea, and stomach cramps. Transmission occurs through the faecal-oral route either by consumption of contaminated water/food or via direct person-to-person contact [93].

1.3.5. Aichivirus

Aichivirus (family *Picornaviridae*, genus *Kobuvirus*) was first isolated in 1989 in a faecal specimen of a patient with nonbacterial gastroenteritis. Aichvirus (AiV) has

a single-stranded positive-sense RNA genome with 8251 bases excluding a poly (A) tail. This genome contains a large ORF with 7302 nucleotides followed by 237 nucleotides of a 3' UTR region and a poly (A) tail. The ORF encodes a polyprotein precursor of 2,432 amino acids with a non-structural leader (L) protein at the N-terminus, followed by viral capsid proteins P1 (VP0, VP3 and VP1) and non-structural proteins P2 (2A,2B and 2C) and P3 (3A,3B,3C and 3D). There are three distinct genotypes of this foodborne virus : AiV A, AiV B and AiV C [94,95].

AiV A and B have been reported on three different continents (Asia, Europe and Africa), mainly in Asian countries (Japan, Vietnam, and Bangladesh), while AiV C has only been reported in France and Burkina Faso [95].

1.4. Laboratory tools used to diagnose pathogens causing AGE

The advancement of diagnostic tools has dramatically changed and improved over the last two decades. Nowadays, molecular methods enable identification of pathogens that may have previously gone undetected. While the relative insensitive and labour-intensive methods (microscopy and cell culture) are largely going to be superseded in routine diagnosis, such new tools are proving particularly valuable for determining the potential effectiveness of certain interventions, such as vaccines.

1.4.1. Cell culture system for enteric virus isolation

Viruses can be grown in vivo (inside a living organism, plant, or animal) or *in vitro* (outside a living organism, in cells in an artificial environment, such as a test tube, cell culture flask or agar plate). Isolation of a virus using cell culture is the most conventional way of confirming the diagnosis. Traditional virus cell culture is considered a relatively slow diagnostic method, as it can take several weeks for the virus to grow. This tool is still the ideal and gold standard method for virus detection worldwide [96].

Traditional cell culture method is suitable for isolation of a wide variety of viruses (including mixed cultures) and provides an isolate for additional studies [97].

1.4.2. Microscopy

1.4.2.1. Identification of *Cryptosporidium*

Conventional methods for the detection of *Cryptosporidium* oocysts include examination of faecal smears with acid-fast stains such as Ziehl-Neelsen, and direct wet mount or by direct immunofluorescence assays (DIFA). More often, oocysts concentrated stool specimens are detectable after staining of the faecal smears. DIFA is more sensitive and specific compared to acid-fast staining [98].

1.4.2.2. Enteric virus detection

The electron microscope (EM) created by Knoll and Ruska in 1932 is an important tool in the diagnosis of enteric virus's infection. This tool allowed the first visualization of enteric virus in 1971. EM is useful in detecting a variety of enteric viruses quickly, but requires high quantities of virus (e.g., > 10^6 viral particles/mL). This tool requires technical expertise, training, and labor costs [97,99].

1.4.3. Immunological methods

The most widespread and simplest serological diagnosis is the enzyme-linked immunosorbent assay (ELISA) either developed as an in-house procedure or as a commercial kit. ELISA, based on antibodies specific to the infectious agent, allows a diagnosis to be made from a single sample [100]. ELISAs can be 10-100 times more sensitive than microscopy.

As with the ELISA methods, there are a range of other antigen-based tests, such as rapid membrane enzyme immunoassays and the latex agglutination test (for enteric viruses). These tests are typically less sensitive than ELISA methods, but yield a result in shorter time and are simple to perform.

The latex agglutination test is one of the most popular, easiest and quickest methods, and is therefore used as a pen test for the identification of several enteric pathogens. This technique has proven to be more specific, more sensitive, faster, and cheaper for Rotavirus detection compared to EM or ELISA [96].

The first antigen capture-based enzyme immunoassay (EIA) was introduced in the 1990s to diagnose *Cryptosporidium* [101].

1.4.4. Molecular methods

Molecular methods largely involve the amplification of nucleic acids by various amplification techniques.

Many kinds of molecular methods have evolved from the classical PCR to meet the demand for highly sensitive and specific diagnostic tests. These different methods include real-time qPCR, loop-mediated isothermal amplification (LAMP), restriction enzyme fragment length polymorphisms (RFLP)-PCR, recombinase polymerase amplification (RPA), reverse transcriptase-qPCR. This scientific progress is fundamental for effective molecular diagnosis. The main advantage of PCR methods is that they are generally more sensitive than traditional methods such as ELISA [96,100].

1.5. Objectives

This thesis aimed to evaluate a point-of-care RDT, as well as to determine the baseline data on molecular and genetic characteristics of potential pathogens associated with diarrheal diseases in children under five years in Lambaréné, Gabon.

The objectives of the work presented in this dissertation are as follows:

1) To determine the prevalence of pathogens found in Gabonese children under five years of age presenting with diarrhea at two hospitals in Lambaréné.

2) To evaluate the performance of CerTest Crypto RDT by a composite reference of qPCR and RFLP-PCR for the detection of *Cryptosporidium* spp. in African children admitted to hospital with diarrhea.

3) To describe the epidemiology and genetic diversity of rotavirus A infecting Gabonese children and examine the antigenic variability of circulating strains in relation to available licenced vaccine strains.

4) To study the genetic diversity of four enteric viruses among Gabonese children below five years of age.

2. RESULTS

2.1. Chapter 1

Prevalence of pathogens in young children presenting to hospital with diarrhea from Lambaréné, Gabon

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Prevalence of Pathogens in Young Children Presenting to Hospital with Diarrhea from Lambaréné, Gabon

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Abstract. Diarrheal disease is the second most frequent cause of mortality in children younger than 5 years worldwide, causing more than half a million deaths each year. Our knowledge of the epidemiology of potentially pathogenic agents found in children suffering from diarrhea in sub-Saharan African countries is still patchy, and thereby hinders implementation of effective preventative interventions. The lack of cheap, easy-to-use diagnostic tools leads to mostly symptomatic and empirical case management. An observational study with a total of 241 participants was conducted from February 2017 to August 2018 among children younger than 5 years with diarrhea in Lambaréné, Gabon. Clinical and demographic data were recorded, and a stool sample was collected. The samples were examined using a commercial rapid immunoassay to detect Rotavirus/adenovirus, conventional bacterial culture for *Salmonella* spp., and multiplex real-time PCR for *Cryptosporidium* spp., *Giardia lamblia*, *Cyclospora cayetanensis*, enterotoxigenic *Escherichia coli* (ETEC), and enteroinvasive *Escherichia coli* (EIEC)/*Shigella*. At least one infectious agent was present in 121 of 241 (50%) samples. The most frequently isolated pathogens were EIEC/*Shigella* and ETEC (54/179; 30.2% and 44/179; 24.6%, respectively), followed by *G. lamblia* (33/241; 13.7%), *Cryptosporidium* spp. (31/241; 12.9%), and Rotavirus (23/241; 9.5%). Coinfection with multiple pathogens was observed in 33% (40/121) of the positive cases with EIEC/*Shigella*, ETEC, and *Cryptosporidium* spp. most frequently identified. Our results provide new insight into the possible causes of diarrheal disease in the Moyen-Ogooué region of Gabon and motivate further research on possible modes of infection and targeted preventive measures.

INTRODUCTION

More than half a million deaths of children younger than 5 years worldwide are caused by diarrheal disease each year, with countries with a lower sociodemographic index disproportionally affected.[1] It is overall the second most frequent cause of death in children aged between 1 and 60 months.[2] Both the diagnosis and treatment of diarrheal disease require substantial improvements toward fulfilling the ambitious sustainable development goals, which call for a reduction of under-five mortality worldwide to no more than 25 deaths per 1,000 live births by 2030.[3]

The management of diarrhea as recommended by the WHO mainly promotes symptomatic case management in children.[4] Although very effective, in many cases, further decreases in morbidity and mortality will rely on more targeted, specific therapies and prevention strategies.

The last few years have seen a number of studies that have examined the spectrum of pathogens responsible for diarrhea in children, the most notable of these being the global enteric multicenter study (GEMS),[5] which revealed a heretofore unexpected spectrum of pathogens, most notably comprising *Cryptosporidium* spp.[6,7] These findings also highlighted the need to update the regional maps of the epidemiology of diarrheal pathogens.

In Gabon, no comprehensive studies on the etiology of infantile diarrhea have been conducted. There are only isolated studies on the bacterial,[8] viral,[9] or very specific parasitic causes of diarrhea, namely, *Cryptosporidium* spp.[10,11] Furthermore, there is only a limited number of studies available on diarrhea in children in the whole of Central African region, especially in the recent literature (two reports from the Central African Republic [CAR][12] and Angola[13]).

Here, we report data from a survey of pathogens found in stool of children younger than 5 years presenting with diarrhea at two hospitals in Lambaréné, a semi-urban town of Gabon.

MATERIALS AND METHODS

Ethics statement. The study protocol was approved by the Institutional Ethical Committee of the Centre de Recherches Médicales de Lambaréné (CERMEL) (CEI-CERMEL: 003/2017). Written informed consent was obtained from parents or legal guardians of all including participants.

Study design. This study was designed as a cross-sectional study and was conducted from February 2017 to August 2018. It is part of a larger cross-sectional study entitled "Genetic determinants for the transmission of *Cryptosporidium parvum/hominis* among humans and animals in Africa," which aimed to identify *Cryptosporidium* transmission networks and reservoirs in Gabon, Ghana, Madagascar, and Tanzania by tracing infected children to their closest human and animal contacts (PMIDs: 32150243 and 32658930).

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Study population and site. Children aged between 0 and 5 years presenting at the outpatient departments of the two main hospitals in Lambaréné, Gabon, the Hôpital Albert Schweitzer, and the Centre Hôspitalier Régional Georges Rawiri de Lambaréné, were screened and included in this study if they presented with diarrhea (defined as the passage of three or more liquid stools within 24 hours during the last 3 days), provided a stool sample, and lived within the study area, which spanned a radius of approximately 20 km around the city of Lambaréné situated in the Moyen-Ogooué region. The region is irrigated by the Ogooué River and its tributaries, with many ponds, lakes, and streams, constituting favorable conditions for waterborne pathogens.

At the time that this study was conducted, the Rotavirus vaccine had not yet been included in the national vaccination programme in Gabon.

Children enrolled in the study. Children presenting at one of two hospitals with diarrhea between February 2017 and February 2018 were invited to participate in the study. The participants came from Lambaréné, the capital of the province of Moyen Ogooué of Gabon, and the surrounding villages, which constitutes a more rural setting with no running water and electricity, and less access to the healthcare system.

Field and laboratory procedures. All personnel involved in the project activities were trained on screening procedures, and recording of demographic and clinical data, sample collection, and transportation to the laboratory. To assess the level of dehydration, we used a clinical dehydration score.[14] A fresh stool sample was provided by each child in a labeled leak-proof stool container within 48 hours after inclusion.

Detection of pathogens was performed by different standard methods[5] and diagnostic kits as described by the manufacturers. In brief, Rotavirus and adenovirus were detected using a rapid diagnostic test (SD Rota/Adeno Rapid; Standard Diagnostics, Hagal-dong, Giheung-gu, Yongin-si, Kyonggi-do, Korea). Enterotoxigenic *Escherichia coli* (ETEC) and enteroinvasive *Escherichia coli* (EIEC)/Shigella were identified by performing a real-time PCR using a specific kit (RIDA®GENE ETEC/EIEC multiplex real-time PCR, Darmstadt, Germany). For *Salmonella* spp., a standard culture was performed.[16,17] Another real-time PCR was used to assess the presence of *Cryptosporidium* spp., *Giardia lamblia*, and *Cyclospora cayetanensis* using specific primers and probes as described elsewhere.[18]

Statistical analysis. Data analysis was performed using *R* version 4.0.2 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria). We calculated the point prevalence of enteric pathogens as the ratio of the number of detected cases per number of patients with evaluable test results for a specific pathogen at the time of presentation to the outpatient or inpatient departments. The assessment of association between categorical variables (or comparison of proportions between different groups: mono-infected, coinfected) was performed with chi-squared test or Fisher's test. For the association between pathogens and age-groups, gender, residence, and odds ratios with 95% CIs were calculated. We calculated binomial 95% CIs for proportions and performed chi-squared tests, and a two-sided *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Baseline characteristics. A total of 329 children presenting at one of the two study hospitals were screened; of these, 241 children were then recruited in the study and gave a stool sample (Supplemental Figure S1 and Table S1); 149 (61.8%) were males and 92 females (38.2%). Sixty-four (27.2%) children were younger than 7 months, 69 (39.4%) were aged between 7 and 12 months, and the remaining 102 (43.4%) were aged between 13 and 59 months. The majority (170/205; 82.9%) of the participants lived in an urbanized area (Table 1).

A total of 199 patients completed the clinical examination. Among those, 63 (31.7%) patients showed no signs of dehydration, whereas 109 (54.8%) were mildly, 26 (13%) moderately, and one (0.5%) child was severely dehydrated. For 137 of 232 participants (59.1%), bloody or mucoid stool was reported, and the most common additional symptoms were, in decreasing order, fever (156/233; 67%), anorexia (141/229; 61.6%), and vomiting (95/215; 44.2%). The number of symptomatic children included in our study varied over time (Supplemental Figure S2).

Detection of enteric pathogens. Of the 241 samples collected, 179 samples could be tested for EIEC/*Shigella* and ETEC using Multiplex real-time PCR, because of an insufficient amount of biological material. No pathogen was identified in 58 children of 179 who provided stool samples (Supplemental Table S1). Thirty percent ($n = 54$) of the samples were positive for EIEC/*Shigella* and 24.6% ($n = 44$) for ETEC.

Giardia lamblia was identified in 13.7% ($n = 33$), *Cryptosporidium* in 12.9% ($n = 31$), and Rotavirus in 9.5% ($n = 23$) of the samples (Table 2).

Proportion of coinfections. Of the 179 samples that had been screened for all the eight studied pathogens, 121 were infected with at least one pathogen. Among these positive samples, 40 (33%) contained more than one pathogen, with dual, triple, and more infections being recorded. Of these 40 stool samples, dual infection was detected in 34 specimens, three pathogens could be simultaneously identified in five

TABLE 1
Characteristics of study participants

Characteristic	*n* (%)	Binomial 95% CI
Age-group (months) (*N* = 235)		
0–6	64 (27.2)	19.8–31.1
7–12	69 (29.4)	24.3–36.2
13–59	102 (43.4)	37.9–50.7
Gender (*N* = 241)		
Female	92 (38.2)	32–44.3
Male	149 (61.8)	55.7–68
Residence (*N* = 205)		
Rural	35 (17.1)	11.9–22.2
Urban	170 (82.9)	77.8–88.1
Vomiting (*N* = 215)		
No	120 (55.8)	49-2-62.5
Yes	95 (44.2)	37.5–50.8
Bloody or mucoid stool (*N* = 232)		
No	95 (40.9)	34.6–47.6
Yes	137 (59.1)	52.4–65.4
Dehydration (*N* = 199)		
None	63 (31.7)	25.2–38.1
Mild	109 (54.8)	47.9–61.7
Moderate	26 (13)	8.4–17.7
Severe	1 (0.5)	0–1.5
Fever (*N* = 233)		
No	77 (33)	27–39.1
Yes	156 (67)	60.9–73
Anorexia (*N* = 229)		
No	88 (38.4)	32.1–44.7
Yes	141 (61.6)	55.3–67.9

TABLE 2
Proportion of pathogens detected in children with diarrhea

Pathogen	*n* (%)	*N* tested
Giardia lamblia		
Positive	33 (13.7)	241
Cryptosporidium spp.		
Positive	31 (12.9)	241
Cyclospora cayetanensis		
Positive	8 (3.3)	241
Rotavirus		
Positive	23 (9.5)	241
Adenovirus		
Positive	8 (3.3)	241
Salmonella spp.		
Positive	5 (2.1)	241
Enterotoxigenic *Escherichia coli*		
Positive	44 (24.6)	179
Enteroinvasive *Escherichia coli*/*Shigella*		
Positive	54 (30.2)	179

samples, and one sample was positive for four pathogens. The most frequently detected pathogens in combination were the two *E. coli* pathotypes (EIEC/*Shigella* [65%] and ETEC [60%]) followed by *Cryptosporidium* spp. (25%), Rotavirus, and *G. lamblia* (22%) (Table 3). Among these, the most frequently observed combinations of pathogens were EIEC/*Shigella* and ETEC, ETEC and Rotavirus, and *Cryptosporidium* and EIEC/*Shigella* (Figure 1).

Association between pathogen carrier status and demographic data. Table 4 summarizes the association between key demographic characteristics (age, gender, and area of residence) of children with diarrhea and potential pathogens causing diarrhea detected in these children. A significant association could be observed for three pathogens and age-group. *Giardia lamblia* was significantly associated with the age-group of 0–6 months (odds ratio [OR]: 0.29; 95% CI: 0.08–0.8, $P = 0.03$) and the age-group of 7–12 months (OR: 0.43; 95% CI: 0.16–1.0, $P = 0.03$). Rotavirus and EIEC/*Shigella* showed, respectively, a significant association with the age-group of 7–12 months (Rotavirus: OR: 0.2; 95% CI: 0.03–0.6, $P = 0.001$; EIEC/*Shigella*: OR: 1.5; 95% CI: 0.6–4.0, $P = 0.02$) and the age-group of 13–59 months (Rotavirus: OR: 0.3; 95% CI: 0.1–0.7, $P = 0.001$; EIEC/*Shigella*: OR: 3.0; 95% CI: 1.3–7.7, $P = 0.02$). The distribution of most pathogens was similar across age-groups and with respect to area of residence, with the exception of Rotavirus, which was more frequently found in infants aged < 6 months and in subjects residing in an urban area. *Giardia lamblia* and ETEC on the other hand were more frequently identified in children older than 6 months.

DISCUSSION

This study was the first of its kind conducted in Lambaréné, Gabon, and is overall one of very few studies conducted on this topic in the Central African region.[8–10,12] The other studies conducted in Gabon, by Koko et al.,[8] Lekana-Douki et al.,[9] and Duong et al.,[10] respectively, focused on specific infectious agents, either bacteria or *Cryptosporidium* spp., and therefore do not provide an epidemiological overview. Furthermore, these studies were conducted mainly in Libreville, a more urbanized setting than Lambaréné.

The most prevalent pathogens in our study were EIEC/*Shigella* and ETEC. This ties in well with the findings of the GEMS multicenter study, in which *Shigella* was identified as the number one pathogen and ETEC ranked fourth.[19] Rota- and adenovirus were the second and third most prevalent enteric pathogens in the GEMS study.

A study conducted in Lambaréné, Gabon, in 1997, analyzing 150 participants' stool for different strains of *E. coli*, had not found EIEC/*Shigella* in any of the samples. Enterotoxigenic *Escherichia coli* was identified in seven samples (4.7%), and EAEC was found in 57 samples (38%).[20] Whether this considerable difference between our respective findings is due to an emergence of new strains of *E. coli* in this region or a different testing protocol cannot be conclusively answered. Samples collected as part of the said study were analyzed in Austria and only submitted for molecular diagnostic testing when the growth of suspect *E. coli* had been shown in culture, whereas in this current study, PCR was conducted regardless of results attained by conventional microbiological culture in our study.

Rotavirus was found in 9.5% of our patients, which is a lower prevalence than expected when reviewing the pertinent literature. This may be due to differing diagnostic methods because most of the other studies used molecular diagnostic methods, which are known to be more sensitive. Reported numbers vary between 27.1% in other parts of Gabon,[9] 25.1% in Angola,[13] and 40.4% in the CAR.[12] Each of these studies was conducted in the same age-group with similar inclusion criteria. The higher prevalence of Rotavirus in younger children with diarrhea as opposed to other pathogens in our study supports similar findings from the GEMS report, as well as the studies by Breurec et al.[12] and Gasparinho et al.[13]

Adenovirus was only identified in 3.3% of our cases, whereas its prevalence in another study conducted in Gabon was 19.6%.[9] Again, this discrepancy may reflect different methods of testing. The original GEMS analysis showed a prevalence of < 5% in diarrheal disease also using a rapid test, whereas a second analysis using molecular techniques

TABLE 3
Prevalence of coinfections

Pathogen	Prevalence among mono-infected subjects ($N = 81$), *n* (%)	Prevalence among coinfected subjects (N = 40), n (%)	*P*-value
Adenovirus	3 (3.7)	3 (7.5)	0.65
Rotavirus	11 (13.6)	9 (22.5)	0.33
G. lamblia	10 (12.3)	9 (22.5)	0.24
Cryptosporidium spp.	6 (7.4)	10 (25)	0.02
C. cayetanensis	1 (1.2)	4 (10)	0.07
Enterotoxigenic *Escherichia coli*	20 (24.7)	24 (60)	< 0.001
Enteroinvasive *Escherichia coli*/*Shigella*	28 (34.6)	26 (65)	0.003
Salmonella spp.	2 (2.4)	2 (5)	0.20

Only data from $n = 179$ with full diagnostics are included.

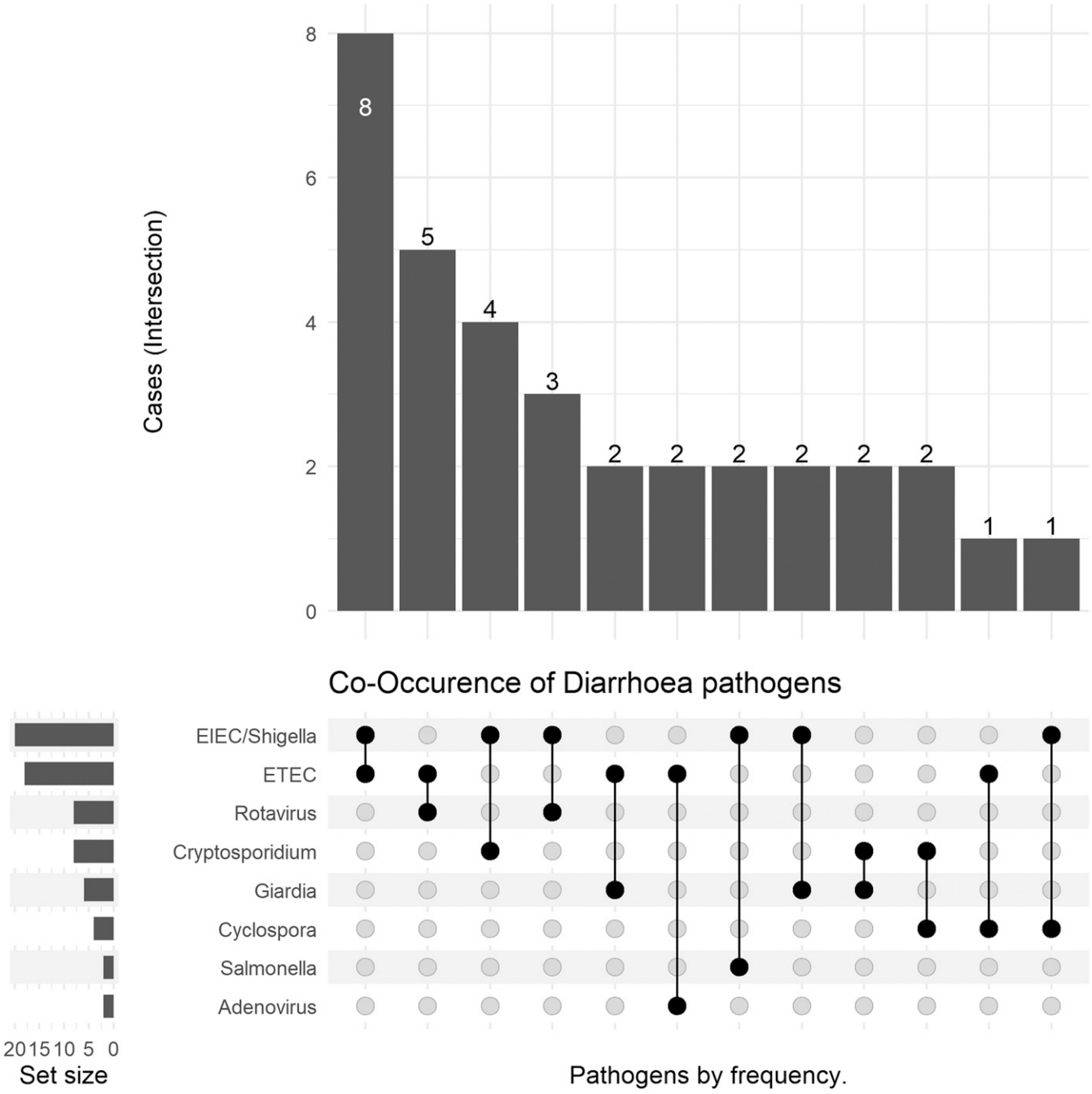

FIGURE 1. Dual pathogen combinations among enteropathogens identified in diarrheal disease.

showed a higher prevalence between 7% and 22% at different sites.[5,19]

The relatively recent discovery of *Cryptopsoridium* spp. as an important pathogen in childhood diarrhea[6] can be further confirmed by our findings (12.9%), with the first cases of *Cryptosporidium* spp. infection recorded in this part of the country.[21] There are estimates that up to 15–25% of children with diarrhea are infected with *Cryptosporidium* spp.[22] A study in Angola found *Cryptosporidium* spp. in 30% of children presenting with diarrhea.[13] Older studies conducted in Kenya and Mozambique found much lower numbers (4% and 0.6%, respectively).[23,24] An earlier study on *Cryptosporidium* spp. in Gabon found a prevalence as high as 24% in children aged between 0 and 2 years with diarrhea.[10] Many of the previous studies used microscopy, which is known to be less sensitive than molecular diagnostic tools such as PCR, leading to the discrepancies for *Cryptosporidium* spp. More generally, the often substantial differences in prevalence estimates stemming from different test technologies appear to be an important limitation for the interpretation of individual studies and hinder potential, more powerful meta-analyses.

The overall findings of this study highlight the need for further examination of the causes of childhood diarrhea and the pathogenesis thereof. Clearly, these results pose a significant question to routinely screen for diarrheagenic *E. coli* such as ETEC and EIEC in this region. Our study showed a high

TABLE 4
Association between enteropathogens and demographic characteristics

Variable	*Rotavirus*, *n* (%)	OR	95% CI	*P*-value	Adenovirus, *n* (%)	OR	95% CI	*P*-value
Age-group (months)								
0–6	13 (22)	1	–	0.001	0 (0)	–	–	0.18
7–12	3 (4)	0.17	0.03–0.56		4 (6)	–	–	–
13–59	7 (7)	0.26	0.09–0.69		3 (3)	1	–	–
Gender								
Female	11 (12)	1	–	0.44	1 (1)	1	–	0.16
Male	12 (8)	0.65	0.27–1.57		7 (5)	3.99	0.68–103	–
Residence								
Rural	2 (6)	1	–	0.54	1 (3)	1	–	1
Urban	19 (11)	1.95	0.52–13.7	–	6 (4)	1.12	0.18–29.6	–
	Enterotoxigenic *Escherichia coli*, *n* (%)	OR	95% CI	*P*-value	*G. lamblia*, *n* (%)	OR	95% CI	*P*-value
Age-group (months)								
0–6	12 (25)	1	–	0.57	4 (7)	0.29	0.08–0.82	0.03
7–12	14 (27)	1.13	0.46–2.84	–	7 (10)	0.43	0.16–1.05	–
13–59	13 (19)	0.72	0.29–1.80	–	21 (21)	1	–	–
Gender								
Female	18 (27)	1	–	0.65	18 (20)	1	–	0.05
Male	26 (23)	0.8	0.40–1.62	–	15 (10)	0.46	0.21–0.96	–
Residence								
Rural	8 (25)	1	–	1	6 (17)	1	–	0.24
Urban	36 (25)	0.97	0.41–2.5	–	17 (10)	0.53	0.20–1.60	–
	Enteroinvasive *Escherichia coli*-*Shigella*, *n* (%)	OR	95% CI	*P*-value	*Salmonella*, *n* (%)	OR	95% CI	*P*-value
Age-group (months)								
0–6	9 (19)	1	–	0.02	1 (2)	0.62	0.02–5.52	0.86
7–12	13 (25)	1.47	0.56–4.00	–	1 (1)	0.52	0.02–4.62	–
13–59	28 (42)	3.05	1.31–7.71	–	3 (3)	1	–	–
Gender								
Female	19 (29)	1	–	0.89	0 (0)	1	–	0.16
Male	35 (31)	1.11	0.57–2.19	–	5 (3)			–
Residence								
Rural	14 (44)	1	–	0.09	1 (3)	1	–	1
Urban	39 (27)	0.47	0.21–1.05	–	4 (2)	0.75	0.10–20.8	–
	Cryptosporidium, *n* (%)	OR	95% CI	*P*-value	*C. cayetanensis*, *n* (%)	OR	95% CI	*P*-value
Age-group (months)								
0–6	3 (5)	1	–	0.11	1 (2)	0.47	0.02–3.50	0.81
7–12	11 (16)	3.33	0.96–16.1	–	3 (4)	1.11	0.20–5.49	–
13–59	16 (16)	3.3	1.03–15.3	–	4 (4)	1	–	
Gender								
Female	13 (14)	1	–	0.79	6 (7)	1	–	0.06
Male	18 (12)	0.83	0.39–1.84	–	2 (1)	0.21	0.03–0.95	–
Residence								
Rural	7 (20)	1	–	0.14	3 (9)	1	–	0.06
Urban	17 (10)	0.44	0.17–1.25	–	3 (2)	0.19	0.03–1.17	–

OR = odds ratio; the reference category is indicated by OR = 1.

number of coinfections, which is similar to the findings of other studies in this field.[5,25–28] The effect of these infections with multiple pathogens on the disease outcome, for example, whether they lead to more severe infection, is as yet not fully understood and calls for more focused research. Furthermore, the fact that some pathogens are more frequently involved in mixed infections could suggest that pathogen combinations can be found in the same contaminated foods or beverages from an area with imperfect sanitation conditions (28). In addition, the high proportion of coinfections could explain the fact that the pathogens detected might act synergistically in pathogenesis and contribute to the burden of diarrheal disease.[29]

The current WHO guidelines on the treatment of infantile diarrhea postulate mainly symptomatic treatment and antibiotic treatment only in cases where amoebiasis or giardiasis is suspected.[4] More easily accessible diagnostic methods and a better knowledge regarding the prevalence of the different pathogens causing diarrhea could help avoid widespread unnecessary prescription of antibiotics.

With regard to the demographic characteristics of the study participants, there were noticeably more male children included (Table 1). Whether this is due to a higher susceptibility to diarrhea, families being more likely to seek professional health care for male children or pure chance cannot be conclusively determined. There was no gender bias in previous hospital-based studies conducted in Lambaréné.[21,30] Overall, the spread of different pathogens was quite even in regard to the participants' demographic characteristics, except for the age of participants. Table 4 shows that most of the pathogens had a higher prevalence in the youngest age-group. This finding is in line with observations made in previous studies

reporting that Rotavirus, *Cryptosporidium*, ETEC, and adenovirus were more frequently detected in children during their first year of life.[5,26] Particularly for children younger than 7 months, we observed a low proportion of most of the enteropathogens found in this study. This could be due to the fact that most children younger than 6 months were usually breastfed, which has been shown to have a preventative effect on gastrointestinal illnesses.[20]

The high number of patients with *G. lamblia* suggests considerable levels of contamination in the local water supply, because this pathogen has been mainly found to be transmitted by water in this demographic group.[31] It is, however, not clear whether this parasite actually always represents a cause of diarrhea or may just be an accompanying colonization as has been suggested by several other authors.[5,12,32,33]

Limitations. The main limitation of this study is the lack of a healthy control group. This excludes any firm attribution of pathogens identified to the symptoms witnessed. Many of these pathogens can also be found in healthy individuals, albeit usually in substantially lower quantity than in those presenting with gastrointestinal symptoms.[19,34,35]

Furthermore, we only tested for certain key pathogens, and thus, alternative pathogenic agents such as *Campylobacter* spp., *Norovirus*, or *Entamoeba histolytica*, to name a few, went undetected. In addition, the methods we used were not necessarily the most sensitive for the identification of the respective pathogens. A study by Li et al. has shown a significantly higher rate of adenovirus identified in samples when using molecular diagnostic methods as opposed to a commercial immunoassay as used in our study.[26] Therefore, we consider the spectrum of pathogens identified in this study as preliminary data that can be used as a baseline for further studies.

CONCLUSION

In this first study of diarrheal pathogens in children in one of the peripheral cities in Gabon and surrounding villages, we identified a spectrum of infectious agents that can serve as baseline for public health stakeholders and guide more detailed future studies. These should include healthy control groups, to enable a more definite attribution of pathogenicity to the different infectious agents identified.

A better understanding of the spectrum of pathogens found in children with diarrhea and more readily available diagnostic methods could lead to a better adherence to the current WHO treatment guidelines and a more appropriate usage of antibiotics by the treating physicians.

Finally, the implementation of the Rotavirus vaccine into the national vaccination program could prove beneficial, particularly for the youngest age bracket examined in this study.

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REFERENCES

1. Troeger C et al., 2017. Estimates of global, regional, and national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet Infect Dis 17:* 909–948.
2. Liu L, Oza S, Hogan D, Chu Y, Perin J, Zhu J, Lawn JE, Cousens S, Mathers C, Black RE, 2016. Global, regional, and national causes of under-5 mortality in 2000–15: an updated systematic analysis with implications for the sustainable development goals. *Lancet 388:* 3027–3035.
3. United Nations, 2015. *Transforming Our World: The 2030 Agenda for Sustainable Development*. United Nations General Assembly 1–35.
4. World Health Organization, 2005. *The Treatment of Diarrhoea: A Manual for Physicians and Other Senior Health Workers*. Geneva, Switzerland: WHO.
5. Kotloff KL et al., 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the global enteric multicenter study, GEMS): a prospective, case-control study. *Lancet 382:* 209–222.
6. Striepen B, 2013. Parasitic infections: time to tackle cryptosporidiosis. *Nature 503:* 189–191.
7. Rossle NF, Latif B, 2013. Cryptosporidiosis as threatening health problem: a review. *Asian Pac J Trop Biomed 3:* 916–924.

8. Koko J, Ambara JP, Ategbo S, Gahouma D, 2013. Épidémiologie des diarrhées aiguës bactériennes de l'enfant à Libreville. *Gabon Arch Pédiatrie 20:* 432–433.
9. Lekana-Douki SE, Kombila-Koumavor C, Nkoghe D, Drosten C, Drexler JF, Leroy EM, 2015. Molecular epidemiology of enteric viruses and genotyping of rotavirus A, adenovirus and astrovirus among children under 5 years old in Gabon. *Int J Infect Dis 34:* 90–95.
10. Duong TH, Kombila M, Dufillot D, Richard-Lenoble D, Owono Medang M, Martz M, Gendrel D, Engohan E, Moreno JL, 1991. Role of cryptosporidiosis in infants in Gabon. Results of two prospective studies. *Bull Soc Pathol Exot 84:* 635–644.
11. Duong TH, Dufillot D, Koko J, Nze-Eyo'o R, Thuilliez V, Richard-Lenoble D, Kombila M, 1995. Cryptosporidiose digestive chez le jeune enfant en zone urbaine au Gabon. *Santé 5:* 185–188.
12. Breurec S et al., 2016. Etiology and epidemiology of diarrhea in hospitalized children from low income country: a matched case-control study in Central African Republic. *PLoS Negl Trop Dis 10:* e0004283.
13. Gasparinho C, Mirante MC, Centeno-Lima S, Istrate C, Mayer AC, Tavira L, Nery SV, Brito M, 2016. Etiology of diarrhea in children younger than 5 years attending the bengo general hospital in Angola. *Pediatr Infect Dis J 35:* e28–e34.
14. Goldman RD, Friedman JN, Parkin PC, 2008. Validation of the clinical dehydration scale for children with acute gastroenteritis. *Pediatrics 122:* 545–549.
15. Savin C, Carniel E, 2009. *Diagnostic des infections*. *Bactériol Méd 2016:* 149–161.
16. Dekker JP, Frank KM, 2015. *Salmonella*, *Shigella*, and yersinia. *Clin Lab Med 35:* 225–246.
17. Verweij JJ, Blangé RA, Templeton K, Schinkel J, Brienen EA, van Rooyen MA, van Lieshout L, Polderman AM, 2004. *Cryptosporidium parvum* in fecal samples by using multiplex real-time PCR. *J Clin Microbiol 42:* 1220–1223.
18. Liu J et al., 2016. Use of quantitative molecular diagnostic methods to identify causes of diarrhoea in children: a reanalysis of the GEMS case-control study. *Lancet 388:* 1291–1301.
19. Presterl E, Zwick RH, Reichmann S, Aichelburg A, Winkler S, Kremsner PG, Graninger W, 2003. Frequency and virulence properties of diarrheagenic *Escherichia coli* in children with diarrhea in Gabon. *Am J Trop Med Hyg 69:* 406–410.
20. Manouana GP et al., 2020. Performance of a rapid diagnostic test for the detection of *Cryptosporidium* spp. in African children admitted to hospital with diarrhea. *PLoS Negl Trop Dis 14:* e0008448.
21. Checkley W et al., 2015. A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for *Cryptosporidium*. *Lancet Infect Dis 15:* 85–94.
22. Gatei W, Wamae CN, Mbae C, Waruru A, Mulinge E, Waithera T, Gatika SM, Kamwati SK, Revathi G, Hart CA, 2006. Cryptosporidiosis: prevalence, genotype analysis, and symptoms associated with infections in children in Kenya. *Am J Trop Med Hyg 75:* 78–82.
23. Mandomando IM et al., 2007. Etiology of diarrhea in children younger than 5 years of age admitted in a rural hospital of Southern Mozambique. *Am J Trop Med Hyg 76:* 522–527.
24. Chen SY, Tsai CN, Chao HC, Lai MW, Lin TY, Ko TY, Chiu CH, 2009. Acute gastroenteritis caused by multiple enteric pathogens in children. *Epidemiol Infect 137:* 932–935.
25. Li LL, Liu N, Humphries EM, Yu JM, Li S, Lindsay BR, Stine OC, Duan ZJ, 2016. Aetiology of diarrhoeal disease and evaluation of viral-bacterial coinfection in children under 5 years old in China: a matched case-control study. *Clin Microbiol Infect 22:* 381.e9–381.e16.
26. Stockmann C et al., 2017. Detection of 23 gastrointestinal pathogens among children who present with diarrhea. *J Pediatr Infect Dis Soc 6:* 231–238.
27. Zhang SX et al., 2016. Impact of co-infections with enteric pathogens on children suffering from acute diarrhea in southwest China. *Infect Dis Poverty 5:* 64.
28. Shrivastava AK, Kumar S, Mohakud NK, Suar M, Sahu PS, 2017. Multiple etiologies of infectious diarrhea and concurrent infections in a pediatric outpatient-based screening study in Odisha, India. *Gut Pathog 9:* 16.
29. Krumkamp R et al., 2020. Transmission of *Cryptosporidium* species among human and animal local contact networks in sub-saharan Africa: a multicountry study. *Clin Infect Dis* ciaa223.
30. Leclerc H, Schwartzbrod L, Dei-Cas E, 2002. Microbial agents associated with waterborne diseases. *Crit Rev Microbiol 28:* 371–409.
31. Nhampossa T et al., 2015. Diarrheal disease in rural Mozambique: burden, risk factors and etiology of diarrheal disease among children aged 0-59 months seeking care at health facilities. *PLoS One 10:* 12–23.
32. Muhsen K, Levine MM, 2012. A systematic review and meta-analysis of the association between *Giardia lamblia* and endemic pediatric diarrhea in developing countries. *Clin Infect Dis 55 (Suppl 4):* S271–S293.
33. Krumkamp R, Sarpong N, Georg Schwarz N, Adelkofer J, Loag W, Eibach D, Matthias Hagen R, Adu-Sarkodie Y, Tannich E, May J, 2015. Gastrointestinal infections and diarrheal disease in Ghanaian infants and children: an outpatient case-control study. *PLoS Negl Trop Dis 9:* e0003728.
34. Platts-Mills JA et al., 2014. Association between stool enteropathogen quantity and disease in Tanzanian children using TaqMan array cards: a nested case-control study. *Am J Trop Med Hyg 90:* 133–138.

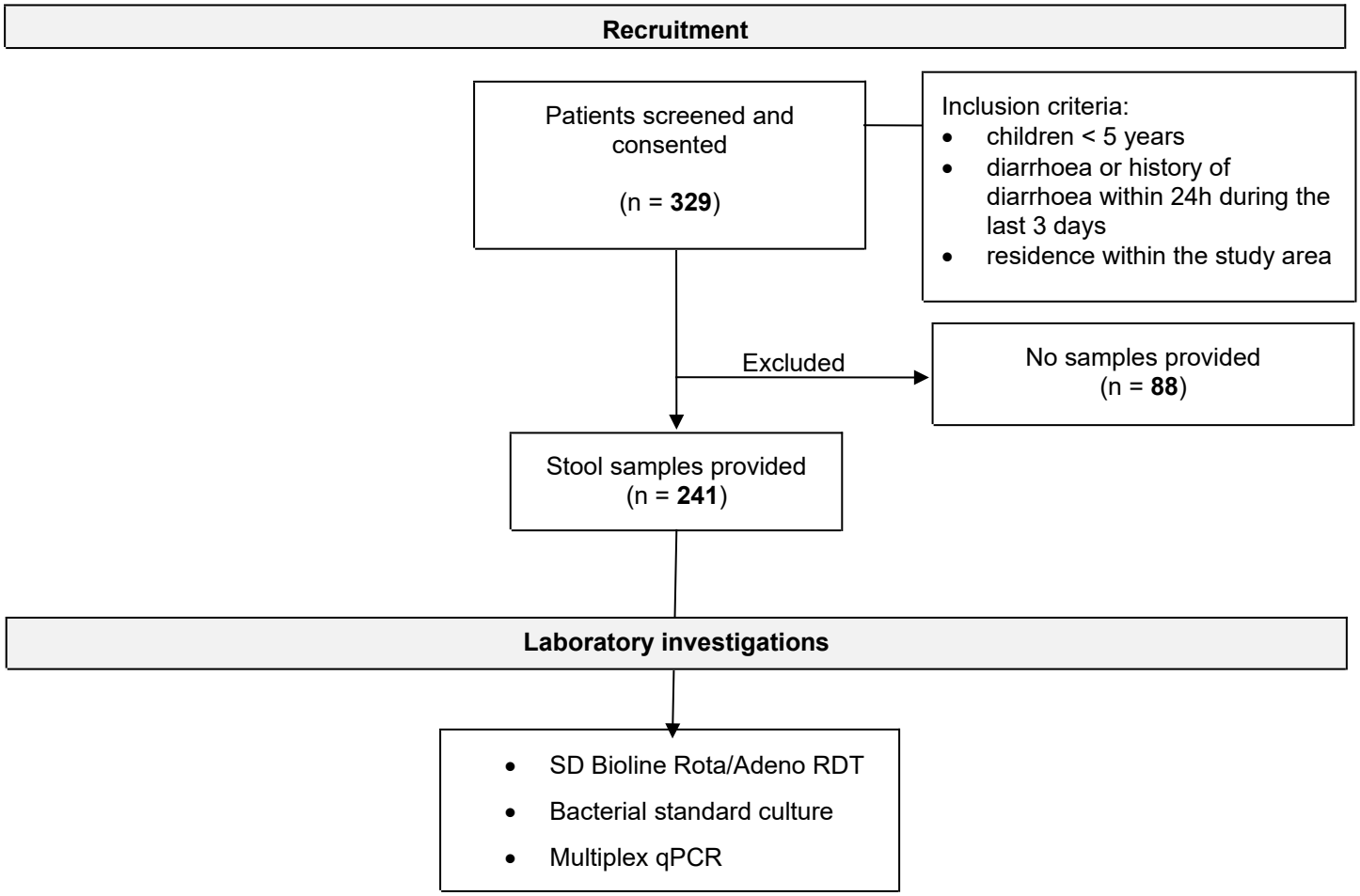
The following are supplemental materials and will be published online only

Supplemental Table S1: Patients recruitment

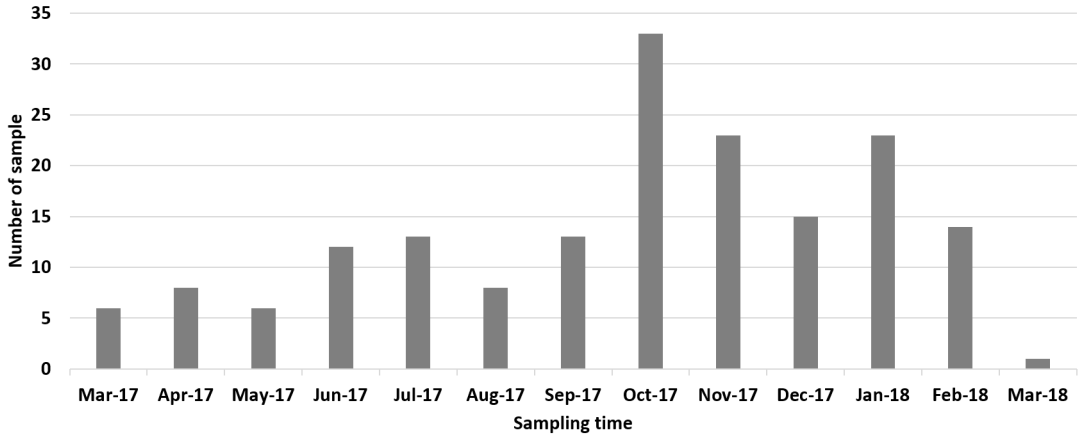
	Included participants	Excluded participants
Number of Patients	241	88
Age in months [median (IQR)]	11.5 (0-59)	14.5 (0-59)
Female sex n (%)	92 (38.2%)	39 (44.3%)

Supplemental Table S2: Fraction of diarrhoea cases with/without a known aetiology by age among the 179 samples screened for all sought pathogens

Age group (Months)	Diarrhoea with unknown etiology	Diarrhoea with known etiology
0-6 (N=55)	26 (47.3%)	29(52.7%)
7-12 (N=53)	19 (35.8%)	34(64.2%)
13-59 (N=71)	13 (18.3%)	58(81.7%)



Supplemental Figure S1: Study workflow diagram



Supplemental Figure S2

2.2. Chapter 2

Performance of a rapid diagnostic test for the detection of Cryptosporidium spp. in African children admitted to hospital with diarrhea

Manouana GP, Lorenz E, Mbong Ngwese M, Nguema Moure PA, Maiga Ascofaré O, Akenten CW, et *al.*

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PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Performance of a rapid diagnostic test for the detection of *Cryptosporidium* spp. in African children admitted to hospital with diarrhea

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Abstract

Background

Cryptosporidium is a protozoan parasite that causes mild to severe diarrhoeal disease in humans. To date, several commercial companies have developed rapid immunoassays for the detection of *Cryptosporidium* infection. However, the challenge is to identify an accurate, simple and rapid diagnostic tool for the estimation of cryptosporidiosis burden. This study aims at evaluating the accuracy of CerTest Crypto, a commercialized rapid diagnostic test (RDT) for the detection of *Cryptosporidium* antigens in the stool of children presenting with diarrhoea.

Methods

A cross-sectional study was conducted in four study sites in Sub-Saharan Africa (Gabon, Ghana, Madagascar, and Tanzania), from May 2017 to April 2018. Stool samples were collected from children under 5 years with diarrhoea or a history of diarrhoea within the last 24 hours. All specimens were processed and analyzed using CerTest Crypto RDT against a composite diagnostic panel involving two polymerase chain reaction (PCR) tests (qPCR and RFLP-PCR,) as the gold standard.

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Competing interests: The authors have declared that no competing interests exist.

Results

A total of 596 stool samples were collected. Evaluation of the RDT yielded a very low overall sensitivity of 49.6% (confidence interval (CI) 40.1–59.0), a specificity of 92.5% (CI 89.8–94.7), positive predictive value of 61.3% (CI 50.6–71.2), and negative predictive value of 88.5% (85.3–91.1) when compared to the composite reference standard of qPCR and RFLP-PCR for the detection of *Cryptosporidium* species. Moreover, the performance of this test varied across different sites.

Conclusion

The weak performance of the studied RDT suggests the need to carefully evaluate available commercial RDTs before their use as standard tools in clinical trials and community survey of *Cryptosporidium* infections in pediatric cohorts.

Author summary

Diarrhoea is a common cause of death among children younger than 5 years. Treatment is based on oral rehydration and sometimes the administration of antibiotics. Several pathogens are responsible for diarrhoea in small children. *Cryptosporidium* species are one of the common pathogens causing prolonged and persistent diarrhoea, malnutrition and growth deficits among immunocompetent children and of severe diarrhoea in immunocompromised persons.

Laboratory diagnosis of cryptosporidiosis is usually achieved by microscopic detection of *Cryptosporidium* oocysts in stool specimens; staining techniques include acid-fast stains and immunofluorescence. Given that appropriate treatment is impeded by the lack of timely and accurate standard diagnostics in middle and low-income countries, rapid diagnostic tests at the point of care potentially offer a shorter time to adequate care. We evaluated a new RDT targeting *Cryptosporidium* species. This new RDT showed variable and insufficient sensitivity among children admitted to a hospital with diarrhea at four different study sites in Gabon, Ghana, Madagascar, and Tanzania.

However, this new RDT could not be used as an appropriate tool for a reliable diagnosis of Cryptosporidiosis to guide community-based screening programs.

Introduction

Diarrheal disease accounts for one in ten cases of death among children younger than five years [1]. Cryptosporidiosis is caused by a coccidian parasite belonging to the genus *Cryptosporidium* and has been recognized as one of the major causes of diarrheal disease worldwide [2]. Several species of *Cryptosporidium* are present in several host where they can cause disease. The First reports of human illness occured in 1976, when *Cryptosporidium* was identified in rectal biopsy specimens of a 3-year-old child [3–4]. *Cryptosporidium parvum and Cryptosporidium hominis* have been reported as the major causes of persistent diarrhea in developing countries, recognized as an opportunistic disease in HIV/AIDS patients, but also responsible for large outbreaks in immunocompetent individuals in developed countries [5–6]. *Cryptosporidium spp* therefore constitute a public health concern particularly due to reports of outbreaks in day care centers, immunocompromised patients and in waterborne transmissions [7–9]. The main symptoms of Cryptosporidiosis include; watery diarrhea which may be profused or prolonged, nausea, vomiting and low-grade fever [10–11]. In developing countries, other

etiology related to the disease includes malnutrition and growth deficits among immunocompetent children and severe diarrhoea in immunocompromised persons [12].

Numerous diagnostic techniques have been used to detect *Cryptosporidium* infection in humans and animals. They include the use of faecal smears stained by the modified Ziehl–Neelsen technique, Enzyme-linked immunosorbent assays (ELISA) and polymerase chain reaction (PCR) assays [13–14]. A comprehensive overview into the detection and molecular characterization of *Cryptosporidium* has been described elsewhere [15]

The above mentioned methods have drawbacks such as being time-consuming and relatively expensive, as well as requiring well-equipped laboratories and well-trained or skilled personnel [14–16].

To overcome these limitations, antigens based-tests mostly relying on immuno-chromatographic assays have been marketed for rapid detection of *Cryptosporidium* antigens. RDTs have become increasingly popular tools and are highly suitable for point of care testing. They allow detection of antigens of one or more protozoan parasites in a single test format by lateral flow immunochromatographic assay. The advantage of such assay is that they are fast, easy to perform and interpret and thus can be used in low resource settings. Several of these RDTs have been in used over the years with varying sensitivities and specificities. In comparing the performance of 4 RDTs including; ImmunoCardSTAT! CGE, Crypto/Giardia Duo-Strip, RIDA QUICK *Cryptosporidium/Giardia/Entamoeba* Combi and *Giardia/Cryptosporidium* Quik Check the authors observed varying sensitivities ranging from 92 to 100% and 100% specificities for all RDT brands for the detection of *Cryptosporidium* when compared to ELISA, microscopy, and qPCR as gold standards [17]. In addition, these RDTs demonstrate varying performances, and some of them are not well validated for the detection of less frequent non-parvum/hominis *Cryptosporidium* species [2–18].

The CerTest Crypto is a newly commercialized RDT with manufacturer reported sensitivity and specificity of >99% respectively [19]. No studies have so far evaluated the performance of this RDT in field settings including resource-limited settings such as ours. More so, most RDTs need to be refrigerated before use, which makes them unsuitable or difficult to use in settings with varying temperatures and humidity. The CerTest Crypto RDT however does not require refrigeration.

Consequently, there is a need for an extensive evaluation of this RDT in diverse field conditions in order to evaluate their diagnostic usefulness. The aim of this study was to assess the performance of CerTest Crypto against a composite reference standard of qPCR and RFLP-PCR.

Materials and methods

Study design, context and site

This prospective cross-sectional study, is part of a larger cross-sectional study entitled "Genetic determinants for transmission of *Cryptosporidium parvum/hominis* among humans and animals in Africa". The study was carried out from May 2017 to April 2018 in four sub-Saharan African countries (Lambaréné in Gabon, Agogo in Ghana, Antananarivo in Madagascar and Korogwe in Tanzania). All hospitals where participants were recruited are situated in semi-urban areas across all study sites. Samples used to evaluate the test performance of CerTest Crypto were collected from these health centers.

Study population

Stool samples were obtained from all children under 5 years of age presenting with diarrhoea or history of diarrhoea within the last 24h to the outpatient departments (OPDs) of the study hospitals of the four countries.

Field and Laboratory procedures

Patients who provided a stool sample and an informed consent signed by the parents or legal guardian were enrolled in the study. Each child provided a fresh stool sample in a dry and leak-proof stool container. Prior to the start of the study, a general training was organized in Tanzania with participants from all the four study sites in attendance. Training was based on CerTest Crypto RDT testing and qPCR for Cryptosporidium. Laboratory technicians from all the four sites were trained on sample processing, time of testing, and protocols were developed and harmonized for use in all the four sites. The Standard Operation Pocedures (SOPs) for sample processing and testing with the CerTest Crypto RDT and qPCR were implemented in all the four sites. A small amount of stool sample (250 mg or 250 μl) was stored at -20˚C for further analyses (with qPCR, RFLP-PCR).

CerTest Crypto RDT processing

Fresh stool samples were tested using a commercially available RDT (CerTest BIOTEC S.L, Pol. Industial Rio Gallego II, Zaragoza Spain). The RDTs were stored at room temperature in all the sites according to manufacturer instructions. The stool samples were collected and transported in cool boxes (4˚C) to the laboratory. All samples were analysed within 24 hours of sample collection. Stool sample aliquots were stored at -80˚C for further analysis. Approximately 125 mg or 125 μl of stool sample was transferred into the diluent provided by the kit manufacturer by use of an applicator stick or pipette. The suspension was homogenized by shaking. Four drops of the diluted fecal material were dispensed into the circular window of the test card. The flow was allowed to run for 10 minutes followed by a visual interpretation. The test results were not recorded later than 10 minutes as instructed by manufacturer [19].

The Certest Crypto Kit is supplied with quality control reagents. The quality control reagent consists of a known positive and negative sample. One negative and one positive control was run once a month in all the four sites and for each new batch of test kits. Additionally, the internal procedural control is included in the test, wherein the appearance of a green line in the control line result window is an indication of correct procedural technique and confirms enough specimen volume was used.

DNA isolation

A modified MO BIO-Qiagen stool DNA extraction protocol was performed incorporating six main steps including: sample treatment, cell lysis, inhibitors removal, DNA binding, a wash step, and DNA elution. Genomic DNA was extracted from 250 mg (or 250 μL) of stool with the use of DNeasy PowerSoil Kit (QIAGEN, Hilden Germany) formerly supplied by MO BIO (MO-BIO Carlsbad, CA, USA) as PowerSoil DNA Isolation Kit. All steps of the DNA extraction were performed following the manufacturer's instructions. The nucleic acids were eluted in 100 μL volume and 5 μL of the extract was used for qPCR.

Real-time PCR

DNA amplification was performed in a Rotor-Gene Q instrument (QIAGEN GmbH, Hilden-Germany) in 25 μL reactions using the HotStart Taq master mix kit (QIAGEN, Germany), 3.5 mM MgCl2, 500 nM forward primer (crypto-F) 5'- CGC TTC TCT AGC CTT TCA TGA -3', 500 nM reverse primer (crypto-R) 5'- CTT CAC GTG TGT TTG CCA AT-3', 175 nM Crypto probe 5'-ROX-CCA ATC ACA GAA TCA TCA GAA TCG ACT GGT ATC–BHQ2-3'. The primer and probe sequences and corresponding assession numbers have been published elsewhere [20]. These primers and probe sequencies are specific for *Cryptosporidium parvum* and

C. hominis, although their efficiency at detecting other *Cryptosporidium* species has not be evaluated.

All samples were tested in duplicate for *Cryptosporidium* species detection employing the following cycling protocol: one cycle at 95˚C for 15 min (polymerase activation), followed by 45 cycles of 95˚C for 15 seconds (denaturation), 67˚C for 30 seconds (annealing) and 72˚C for 30 seconds (extension), followed by a final cooling step at 40˚C for 30 seconds. Phocin Herpes Virus (PhHV) Plasmid was incorporated into the master mix to control for PCR inhibitors and only samples with a cycle threshold < = 38 were considered positive.

Cryptosporidium genotyping

All samples detected positive for *Cryptosporidium* species by qPCR were genotyped using a PCR–restriction fragment length polymorphism (RFLP) technique as described previously [21]. To improve specificity, the PCR products from the restriction digest were sequenced and resulting sequences were blasted to identify the *Cryptosporidium* species.

Statistical analysis

Statistical analyses were performed using Stata 14. Categorical variables were described as counts and percentages. Continuous variables were described by the median and interquartile range (IQR). To ensure a comparable set of observations for the analysis, observations with missing information on either the RDT or the PCR result were excluded from the analysis and the study period was restricted from May 2017 to April 2018. We calculated sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) to evaluate the performance of the CerTest Crypto RDT result versus the overall results observed by combining two PCR methods (PCR, RFLP-PCR) defined as a composite reference standard PCR. The performance characteristics of CerTest Crypto RDT were determined based on the ability of the CerTest to detect all species of *Cryptosporidium* (both human and animal species). Therefore, to assess the performance characteristics of this test to detect 2 of the most frequent species(*C. hominis* and *C. parvum*), eight observations that were PCR positive for "*C. meleagridis* "and "*C. xiaoi*/*bovis*" were excluded from the analysis. PCR was positive if either method yielded a positive result, otherwise negative. The test performance measures are presented as percentages along with the respective 95% confidence intervals (CI).

Ethical approval

Informed consent was obtained from the parents or legal guardian of the patient at the outpatient department (OPD) or inpatient department (IPD). The study protocol was approved by each Institutional Ethical Review Board of all four study sites. The National Health Research Ethics Committee (NatHREC) in Tanzania, Comité National d'Ethique du Gabon, the Committee On Human Research, Publications And Ethics, Kwame Nkrumah University Of Science And Technology of Kumasi, Ghana, the Medical Research Coordinating Committee of the National Institute for Medical Research, Tanzania (NIMR MRCC), the Ethical Committee of the Ministry of Health of the Republic of Madagascar and the Ärztekammer Hamburg, Hamburg, Germany.

Results

A total of 596 stool samples from Ghana (N = 132), Gabon (N = 192), Madagascar (N = 83) and Tanzania (N = 189) was tested to assess the performance of CerTest Crypto RDT. Table 1 provides the frequency distribution of all *Cryptosporidium* species from all study sites

Table 1. Frequency distribution of *Cryptosporidium* rapid diagnostic test results by study sits.

Country	C. hominis n (%)	C. meleagridis n (%)	C. parvum n (%)	C. xiaoi/bovis n (%)
Overall (N = 482)	91 (18.9)	7 (1.5)	15 (3.1)	1 (0.2)
Ghana (N = 107)	15 (14)	2 (1.9)	7 (6.5.)	1 (0.9)
Gabon (N = 154)	31 (20.1)	0	7 (4.5)	0
Madagascar (N = 65)	14 (21.5)	4 (6.2)	0	0
Tanzania (N = 156)	31 (19.9)	1 (0.6)	1 (0.6)	0

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following analysis with PCR and RFLP. The diagnostic performance characteristics of *Cryptosporidium* CerTest Crypto RDT were analyzed using PCR as a reference standard in all 4 study sites. Table 2 provides sensitivity, specificity, PPV, and NPV of the test to detect *Cryptosporidium* species in the four sites. The sensitivity of the test varied considerably in all study sites being highest in Madagascar (72.22%) in comparison to Gabon and Ghana which had 50 and 52% respectively and was lowest in Tanzania with 35.29%. Conversely, the specificity of the test was similar in all study sites ranging from 86 to 94%. However, subsequent re-analyses did not result in any changes in the performance characteristic of the cerTest across study sites (Table 3).

Further analyses of all cerTest negative samples from all study sites was performed using PCR-RFLP.

To further evaluate the stability throughout the year and batches variabilities of the CerTest Crypto against PCR reference standard, we looked at the performance of the test over the course of the year in all study sites (Figs 1–4). Overall the false netative (FN) cases were detected almost every month in Gabon from the start to the end of the study period. Meanwhile, in Ghana, FN cases were detected more frequently during the first 5 months (May to September 2017) and then during the last 2 months (March to April 2018). While in Madagascar and Tanzania, the FN cases were mostly found in the beginning and towards the end of the study period. These numbers of FN observed at the four sites are not consistent and do not represent a clear pattern to suggest any batch effect on the test performance from the different study sites.

Finally, there is no evidence of the effect of gender, rainfall, sampling period as well as age group on *Cryptosporidium* infection across the study sites. However, there is a high proportion of *Cryptosporidium* infection occurring during the first two years of age accros countries (S1 Table and S1 Fig).

Discussion

There is a need for efficient diagnostic methods for *Cryptosporidium* infection in settings where the disease is prevalent. Consequently, RDTs appear as a unique opportunity for point-of-care diagnosis in the absence of routine stool microscopy and advanced diagnostic tools. CerTest Crypto test is a commercially available RDT that is easy to perform, does not require

Table 2. Overall and country specific test evaluation results of the RDT when compared to qPCR and RFLP as reference standard.

Country	Sensitivity [95% CI]	Specificity [95% CI]	Positive predictive value [95% CI]	Negative predictive value [95% CI]
Overall (N = 596)	49.57 [40.11, 59.04]	92.52 [89.79, 94.70]	61.29 [50.62, 71.22]	88.47 [85.35, 91.13]
Ghana (N = 132)	52.00 [31.31, 72.20]	94.39 [88.19, 97.91]	68.42 [43.45, 87.42]	89.38 [82.18, 94.39]
Gabon (N = 192)	50.00 [33.38, 66.62]	94.81 [90.02, 97.73]	70.37 [49.82, 86.25]	88.48 [82.60, 92.92]
Madagascar (N = 83)	72.22 [46.52, 90.31]	86.15 [75.34, 93.47]	59.09 [36.35, 79.29]	91.80 [81.90, 97.28]
Tanzania (N = 189)	35.29 [19.75, 53.51]	91.61 [86.08, 95.46]	48.00 [27.80, 68.69]	86.59 [80.40, 91.40]

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Table 3. Overall and country specific test evaluation results of the RDT when compared to qPCR and RFLP as reference standard, excluding test results of *Cryptosporidium* meleagridis", "*Cryptosporidium* xiaoi/bovis".

Country	Sensitivity [95% CI]	Specificity [95% CI]	Positive predictive value [95% CI]	Negative predictive value [95% CI]
Overall (N = 588)	49.53 [39.72, 59.37]	92.52 [89.79, 94.70]	59.55 [48.62, 69.83]	89.18 [86.12, 91.77]
Ghana (N = 129)	54.55 [32.21, 75.61]	94.39 [88.19, 97.91]	66.67 [40.99, 86.66]	90.99 [84.06, 95.59]
Gabon (N = 192)	50.00 [33.38, 66.62]	94.81 [90.02, 97.73]	70.37 [49.82, 86.25]	88.48 [82.60, 92.92]
Madagascar (N = 79)	71.43 [41.90, 91.61]	86.15 [75.34, 93.47]	52.63 [28.86, 75.55]	93.33 [83.80, 98.15]
Tanzania (N = 188)	36.36 [20.40, 54.88]	91.61 [86.08, 95.46]	48.00 [27.80, 68.69]	87.12 [80.98, 91.84]

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refrigeration and saves time for the detection of *Cryptosporidium* spp. According to previous studies, the performance levels' variability of RDTs can depend on differences in commercial products, dissimilar methodologies employed and genetic diversity of *Cryptosporidium* across geographical regions [18]. The cerTest Crypto RDT, whose high-performance parameters (Sensitivity and specificity > 99%) has been claimed by the manufacturer, was evaluated in this study. Our observations on the performance characteristics of cerTest Crypto showed high specificity (Tables 1 and 2). Our findings suggest that this RDT is reliable for detecting samples with no *Cryptosporidium*. As mentioned in previous studies, this result could also mean no cross-reaction with other pathogens causing diarrhoea [18–22].

In contrast, there are different sensitivities in each study site, ranging from 35.29% to 72.22% for the detection of *Cryptosporidium* species and from 36.36% to 71.43% for the detection of *C. parvum* and *C. hominis*. This indicates that this test performs poorly in the detection of all *Cryptosporidium* species. The overall number of true positives (TP) was generally low for all four sites. Meanwhile, the number of false negative did not vary considerably by month. The trend of the distribution of *Cryptosporidium spp*. that we observed amongst the younger children is similar to that reported by Current and Garcia in 1991 [23], suggesting that the demographics could not influence the performance characteristics of cerTest Crypto (S1 Table).

Therefore, the observed low sensitivity could be due to several other factors such as low parasite densities as reported elsewhere [24–25]. Moreover, our findings show a lower apparent sensitivity of the test kit that may be as a result of comparing the test to PCR, which can detect

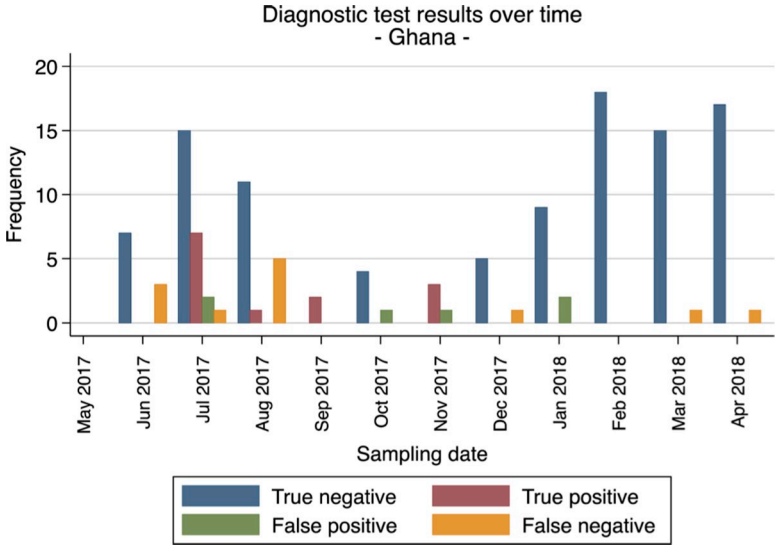

Fig 1. Frequencies of diagnostic test evaluation (RDT vs. PCR as reference standard) from all patients for Ghana.

https://doi.org/10.1371/journal.pntd.0008448.g001

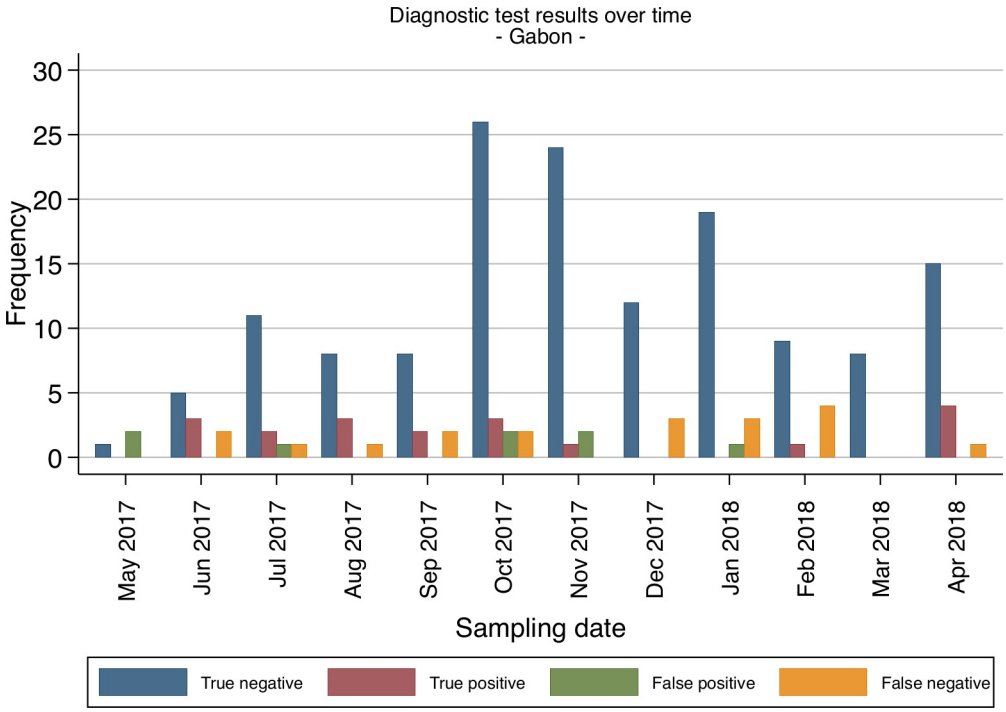

Fig 2. Frequencies of diagnostic test evaluation (RDT vs. PCR as reference standard) from all patients for Gabon.

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parasite DNA remaining in a patient's after an infection has been cleared as previously mentioned by Boyce and O'Meara in 2017 [24]. Contrary to the study conducted by Bouyou-Akotet [25] in which the authors suggest that low sensitivity was correlated to the lower parasite load in a test that targets a specific species [26], our finding revealed a lower overall sensitivity with CerTest RDT which was not specific to one particular species.

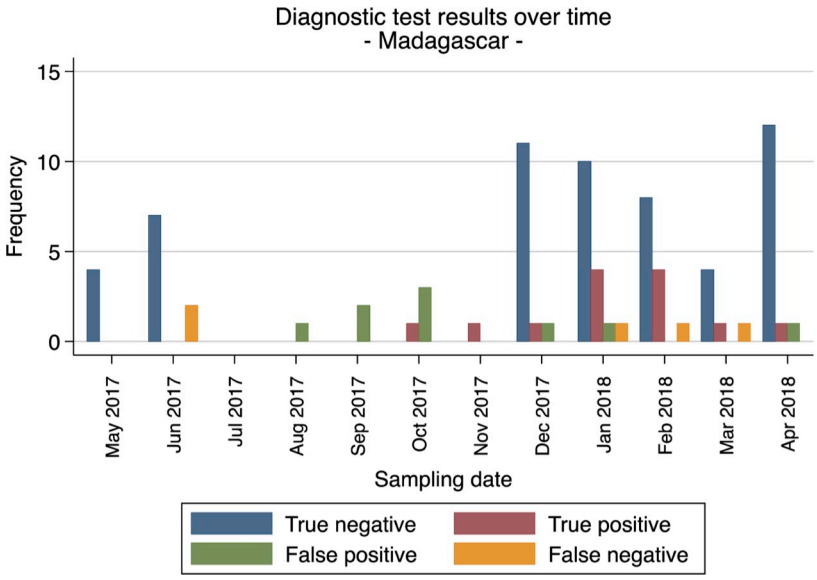

Fig 3. Frequencies of diagnostic test evaluation (RDT vs. PCR as reference standard) from all patients for Madagascar.

https://doi.org/10.1371/journal.pntd.0008448.g003

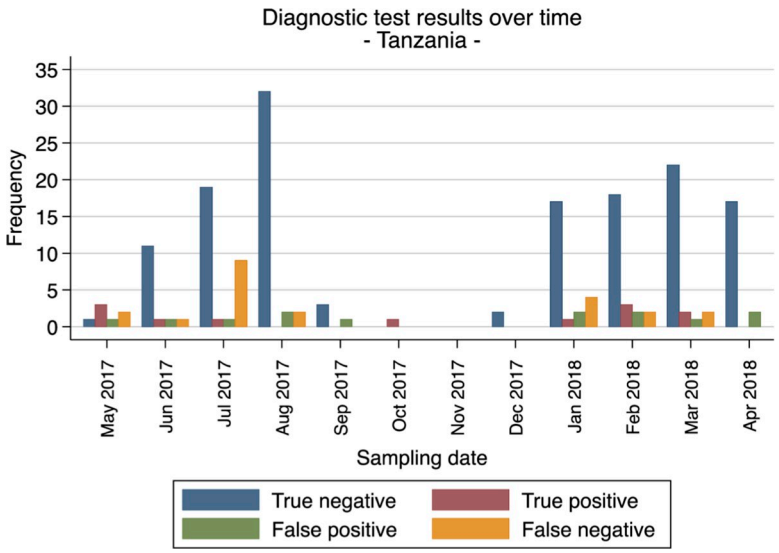

Fig 4. Frequencies of diagnostic test evaluation (RDT vs. PCR as reference standard) from all patients for Tanzania.

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Furthermore, our finding highlighted the overall decrease in PPV due to the very low number of TP observed from the four sites. Although the NPV of 88.47% can be considered high enough, this is not comparable to manufacturer reported > 99% NPV. A considerable number of FN was observed from all study sites. Without any microscopy data to correlate the observed oocyst concentrations (infection intensities) and percentage of FN, it is difficult to determine the effect of parasite load on the detection of all species of *Cryptosporidium* (Tables 1 and 2). More so, some *Cryptosporodium* genotypes may lack part or all of the protein targeted by the CerTest RDT which could explain the observed numbers of FN. Additionally, the PPV of 61.29% is due to very low numbers of TP and a considerable number of FP observed from all sites.

The evaluation of the sensitivity of RDTs used in the detection of malaria parasites has been reported to be influenced by changing seasons [27]. In light of this, the observed differences in our case could be correlated with the fluctuation of temperatures in all study areas. Thus, most cases of FN and FP found in different months were likely due to the incidence of Cryptosporidiosis that varies with changing seasons and across geographical areas. Moreover, other reports suggest that temperatures above 30˚C can affect the overall performance of the RDTs [28] in the case of malaria parasites detection. Taken together, the accuracy of any RDT result may depend on several factors such as the quality of the RDT, storage, transport and end-user performance.

Our study had two main limitations. Firstly, due to the absence of microscopy data on oocyst concentrations, we could not correlate *Cryptosporidium* oocyst load and the test performance. However, according to the manufacturer, the current test does not detect low concentration antigens in the stool. Hence a sample that tests negative with the RDT is unlikely to be positive microscopically. Taken together, we could not report the cause of all FN reported from all study sites. Secondly, with no additional investigation on protozoan pathogens causing diarrhea, we cannot exclude the possibility of cross-reacting species as the cause of all FP cases in this study. Our demographic data did not not provide any substantial evidence on the effect of temperature and humidity on the test kit. Thus the variation in performance of the test kit that we observed in this study is not related to the handling and storage of the kits.

In conclusion, CerTest Crypto RDT has been designed to detect *Cryptosporidium species*. Although this coproantigen detection assay is rapid, its sensitivity is low for the detection of *Cryptosporidium* spp as well as *C. parvum* and *C. hominis* in particular. Therefore, additional research is needed to evaluate the performance of the CerTest Crypto RDT with particular emphasis on light intensity infections whereby the concentrations of antigens present in the sample are below the detection limit of the test that may result in an increase number of false negatives.

Supporting information

S1 Checklist. STARD-checklist for CerTest Crypto RDT study.
(DOCX)

S1 Diagram. STARD diagram for participants flow in CerTest Crypto RDT study.
(PNG)

S1 Data. Data base_comb_reduced.
(DTA)

S1 Table. Proportion of *Cryptosporidium*-PCR positives and demographic data across the four study sites.
(DOCX)

S2 Table. Proportion of *Cryptosporidium*-PCR positives and demographic data across the four study sites.
(DOCX)

S1 Fig. Distribution of infected stools across sites and age in months.
(DOCX)

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References

1. Kotloff K. L. et al., "Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): A prospective, case-control study," *Lancet*, vol. 382, no. 9888, pp. 209–222, 2013. https://doi.org/10.1016/S0140-6736(13)60844-2 PMID: 23680352
2. Goudal A. et al., "Rapid diagnostic tests relying on antigen detection from stool as an efficient point of care testing strategy for giardiasis and cryptosporidiosis? Evaluation of a new immunochromatographic duplex assay," *Diagn. Microbiol. Infect. Dis.*, vol. 93, no. 1, pp. 33–36, 2019. https://doi.org/10.1016/j.diagmicrobio.2018.07.012 PMID: 30122511
3. Garcia L. S. et al., "Laboratory Diagnosis of Parasites from the Gastrointestinal Tract," *Clin. Microbiol. Rev.*, vol. 31, no. 1, pp. e00025–17, Nov. 2017. https://doi.org/10.1128/CMR.00025-17 PMID: 29142079
4. Holscher M. A., Ph D., and Yardley J. H., "ACUTE ENTEROCOLITIS IN A HUMAN BEING INFECTED WITH THE PROTOZOAN CRYPTOSPORIDIUM," *Gastroenterology*, vol. 70, no. 4, pp. 592–598, 1976. PMID: 815126
5. Gertler M. et al., "Outbreak of Cryptosporidium hominis following river flooding in the city of Halle (Saale), Germany, August 2013.," *BMC Infect. Dis.*, vol. 15, p. 88, Jan. 2015. https://doi.org/10.1186/s12879-015-0807-1 PMID: 25879490
6. Mac Kenzie W. R. et al., "A massive outbreak in Milwaukee of cryptosporidium infection transmitted through the public water supply.," *N. Engl. J. Med.*, vol. 331, no. 3, pp. 161–7, Jul. 1994. https://doi.org/10.1056/NEJM199407213310304 PMID: 7818640
7. Tzipori S., Smith M., Birch C., Barnes G., and Bishop R., "Cryptosporidiosis in Hospital Patients with Gastroenteritis," *Am. J. Trop. Med. Hyg.*, vol. 32, no. 5, pp. 931–934, 1983. https://doi.org/10.4269/ajtmh.1983.32.931 PMID: 6625074
8. Karanis P., Kourenti C., and Smith H., "Waterborne transmission of protozoan parasites: A worldwide review of outbreaks and lessons learnt," *J. Water Health*, vol. 5, no. 1, pp. 1–38, Sep. 2006.
9. Bouzid M., Hunter P. R., Chalmers R. M., and Tyler K. M., "Cryptosporidium pathogenicity and virulence," *Clin. Microbiol. Rev.*, vol. 26, no. 1, pp. 115–134, Jan. 2013. https://doi.org/10.1128/CMR.00076-12 PMID: 23297262
10. Current W. L. and Garcia L. S., "Cryptosporidiosis," *Clin. Microbiol. Rev.*, vol. 4, no. 3, pp. 325–358, Jul. 1991. https://doi.org/10.1128/cmr.4.3.325 PMID: 1889046
11. Meinhardt P. L., Casemore D. P., and Miller K. B., "Epidemiologic Aspects of Human Cryptosporidiosis and the Role of Waterborne Transmission," *Epidemiol. Rev.*, vol. 18, no. 2, pp. 118–136, Jul. 1996. https://doi.org/10.1093/oxfordjournals.epirev.a017920 PMID: 9021307
12. Checkley W. et al., "A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for cryptosporidium.," *Lancet. Infect. Dis.*, vol. 15, no. 1, pp. 85–94, Jan. 2015. https://doi.org/10.1016/S1473-3099(14)70772-8 PMID: 25278220
13. Ezzaty Mirhashemi M., Zintl A., Grant T., Lucy F. E., Mulcahy G., and De Waal T., "Comparison of diagnostic techniques for the detection of Cryptosporidium oocysts in animal samples," *Exp. Parasitol.*, vol. 151–152, pp. 14–20, 2015. https://doi.org/10.1016/j.exppara.2015.01.018 PMID: 25662435
14. Papini R., Bonelli F., Montagnani M., and Sgorbini M., "Evaluation of three commercial rapid kits to detect Cryptosporidium parvum in diarrhoeic calf stool," *Ital. J. Anim. Sci.*, vol. 17, no. 4, pp. 1059–1064, Oct. 2018.
15. Cunha F. S., Peralta J. M., and Peralta R. H. S., "New insights into the detection and molecular characterization of Cryptosporidium with emphasis in Brazilian studies: A review," *Rev. Inst. Med. Trop. Sao Paulo*, vol. 61, no. January, pp. 1–12, 2019.
16. Manser M. et al., "Detection of Cryptosporidium and Giardia in clinical laboratories in Europe—a comparative study," *Clin. Microbiol. Infect.*, vol. 20, no. 1, pp. O65–O71, 2014. https://doi.org/10.1111/1469-0691.12297 PMID: 24033667

17. Van den Bossche D., Cnops L., Verschueren J., and Van Esbroeck M., "Comparison of four rapid diagnostic tests, ELISA, microscopy and PCR for the detection of Giardia lamblia, Cryptosporidium spp. and Entamoeba histolytica in feces," *J. Microbiol. Methods*, vol. 110, pp. 78–84, 2015. https://doi.org/10.1016/j.mimet.2015.01.016 PMID: 25615719

18. Shimelis T. and Tadesse E., "Performance evaluation of point-of-care test for detection of Cryptosporidium stool antigen in children and HIV infected adults," *Parasit. Vectors*, vol. 7, no. 1, p. 227, 2014.

19. CerTest BIOTEC SL, "CERTEST Crypto: One Step test to detect Cryptosporidium in card format."

20. Verweij J. J. et al., "Simultaneous detection of Entamoeba histolytica, Giardia lamblia, and Cryptosporidium parvum in fecal samples by using multiplex real-time PCR," *J. Clin. Microbiol.*, vol. 42, no. 3, pp. 1220–1223, Mar. 2004. https://doi.org/10.1128/jcm.42.3.1220-1223.2004 PMID: 15004079

21. Lal A. A. et al., "Identification of 5 Types of Cryptosporidium Parasites in Children in Lima, Peru," *J. Infect. Dis.*, vol. 183, no. 3, pp. 492–497, Feb. 2001. https://doi.org/10.1086/318090 PMID: 11133382

22. Weitzel T., Dittrich S., Möhl I., Adusu E., and Jelinek T., "Evaluation of seven commercial antigen detection tests for Giardia and Cryptosporidium in stool samples," *Clin. Microbiol. Infect.*, vol. 12, no. 7, pp. 656–659, 2006. https://doi.org/10.1111/j.1469-0691.2006.01457.x PMID: 16774562

23. Current W. L. and Garcia L. S., "Cryptosporidiosis," vol. 4, no. 3, pp. 325–358, 1991. https://doi.org/10.1128/cmr.4.3.325 PMID: 1889046

24. Boyce M. R. and O'Meara W. P., "Use of malaria RDTs in various health contexts across sub-Saharan Africa: a systematic review," *BMC Public Health*, vol. 17, no. 1, p. 470, May 2017. https://doi.org/10.1186/s12889-017-4398-1 PMID: 28521798

25. Bouyou-Akotet M. K. et al., "Low sensitivity of the ImmunocardSTAT(®) Crypto/Giardia Rapid Assay test for the detection of Giardia and Cryptosporidium in fecal samples from children living in Libreville, Central Africa," *J. Parasit. Dis.*, vol. 40, no. 4, pp. 1179–1183, Dec. 2016. https://doi.org/10.1007/s12639-015-0645-5 PMID: 27876910

26. Llorente M. et al., "Evaluation of an Immunochromatographic Dip-Strip Test for the Detection of Cryptosporidium Oocysts in Stool Specimens," *Eur. J. Clin. Microbiol. Infect. Dis.*, vol. 21, no. 8, pp. 624–625, 2002. https://doi.org/10.1007/s10096-002-0778-1 PMID: 12226697

27. Bisoffi Z. et al., "Accuracy of a rapid diagnostic test on the diagnosis of malaria infection and of malaria-attributable fever during low and high transmission season in Burkina Faso," *Malar. J.*, vol. 9, p. 192, Jul. 2010. https://doi.org/10.1186/1475-2875-9-192 PMID: 20609211

28. Moonasar D., Goga A. E., Frean J., Kruger P., and Chandramohan D., "An exploratory study of factors that affect the performance and usage of rapid diagnostic tests for malaria in the Limpopo Province, South Africa," *Malar. J.*, vol. 6, p. 74, Jun. 2007. https://doi.org/10.1186/1475-2875-6-74 PMID: 17543127

2.3. Chapter 3

Molecular surveillance and genetic divergence of rotavirus A antigenic epitopes in Gabonese children with acute gastroenteritis

Manouana GP, Niendorf S, Tomazatos A, Mbong Ngwese M, Nzamba Maloum M, Nguema Moure PA, et *al.*,

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Molecular surveillance and genetic divergence of rotavirus A antigenic epitopes in Gabonese children with acute gastroenteritis

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ABSTRACT

Background: Rotavirus A (RVA) causes acute gastroenteritis in children <5 years of age in sub-Saharan Africa. In this study, we described the epidemiology and genetic diversity of RVA infecting Gabonese children and examined the antigenic variability of circulating strains in relation to available vaccine strains to maximize the public health benefits of introducing rotavirus vaccine through the Expanded Programme on Immunization (EPI) in Gabon.

Methods: Stool samples were collected consecutively between April 2018 and November 2019 from all hospitalized children <5 years with gastroenteritis and community controls without gastroenteritis. Children were tested for rotavirus A by quantitative RT-PCR and subsequently sequenced to identify circulating rotavirus A genotypes in the most vulnerable population. The VP7 and VP4 (VP8*) antigenic epitopes were mapped to homologs of vaccine strains to assess structural variability and potential impact on antigenicity.

Findings: Infections were mostly acquired during the dry season. Rotavirus A was detected in 98/177 (55%) hospitalized children with gastroenteritis and 14/67 (21%) of the control children. The most common RVA genotypes were G1 (18%), G3 (12%), G8 (18%), G9 (2%), G12 (25%), with G8 and G9 reported for the first time in Gabon. All were associated either with P[6] (31%) or P[8] (38%) genotypes. Several non-synonymous substitutions were observed in the antigenic epitopes of VP7 (positions 94 and 147) and VP8* (positions 89, 116, 146 and 150), which may modulate the elicited immune responses.

Interpretation: This study contributes to the epidemiological surveillance of rotavirus A required before the introduction of rotavirus vaccination in the EPI for Gabonese children.

1. Introduction

Diarrheal diseases remain one of the major cause of illness among children <5 years, causing over 500,000 deaths worldwide each year, mainly in Africa and South Asia [1]. One of the most important etiological agents of gastroenteritis in infants and children is group A rotavirus (RVA). The non-enveloped, triple-layered viral particle has

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Research in context

Evidence before this study

Rotavirus is one of the most important etiologic agents of infantile gastroenteritis, with an estimated 200,000 deaths in children <5 years of age. There are four WHO prequalified Rotavirus vaccines available thus far. We searched PubMed for publications until 2020 using the search terms "rotavirus A" AND "epitopes" AND "children." We found 10 results describing different studies of rotavirus genetic diversity and characterization of the VP4 and VP7 genes encoding the outer capsid proteins. Eight of the studies compared the antigenic regions of the VP7 and VP4 partial sequences of circulating rotavirus strains with those of Rotarix and RotaTeq vaccines. However, there are no studies from Central Africa, where the Expanded Programme on Immunization (EPI) is scheduled to introduce rotavirus vaccine, particularly in the Republic of Gabon.

Added value of this study

Currently, there is limited data on the genetic similarity of vaccine strains and their relationship with wild-type strains. In this study, we found a high RVA burden in Gabonese children with high antigenic variability in circulating compared to the vaccine strains. This may influence efficacy, as this extensive genetic variability observed in these viruses may evade immune responses induced by prior infection or vaccination through changes in molecular structures by antibodies and/or T cells.

Implications of all the available evidence

The high RVA burden implies an urgent need for the introduction of RVA vaccination for Gabonese children.

a genome of 11 double-stranded RNA (dsRNA) encoding six structural (VP1-VP4, VP6, VP7) and six non-structural (NSP1-NSP6) proteins [2]. The outer capsid layer is formed by the VP7 glycoprotein (G) and the VP4 spike protease-sensitive (P) protein, both encoded by genomic segments on which a binary taxonomical system of rotaviruses is based at intraspecific level, determining their classification into G and P genotypes [3].

The Rotavirus classification work group of the International Committee on Virus Taxonomy has currently identified 41 G and 57 P genotypes in mammal and bird species worldwide [3,4], although in humans only a few genotypes are responsible for the disease burden (G1-G4, G9, G12, P[4], P[6], P[8]) [5–8]. Additionally, genotype G5, G6 and G8 are considered relevant to human health in Africa and Asia [7]. In addition to rapid genetic drift caused by the error-prone RNA-dependent RNA polymerase, segment reassortment (genetic shift) is another major driver of rotavirus evolution, leading to new genetic combinations and enabling interspecies transmission [9,10]. Previous studies reported G1P[8] as the most frequent RVA strain globally [5,11], while G2P[4], G3P[8], G4P[8], and G9P[8] were reported to be common strains worldwide in the pre-vaccination era [11].

There are four WHO prequalified Rotavirus vaccines available thus far. Rotarix (GlaxoSmithKline Biologicals, Belgium) is a monovalent vaccine derived from a human G1P[8] strain and RotaTeq (Merck&Co., USA) is a pentavalent (G1-G4 and P[8]) containing five human-bovine RVA reassortant strains [10]. Both vaccines were proven to be safe and efficacious. While Rotarix and Rotateq are used in over 90 countries worldwide [12], WHO recently prequalified Rotavac (Bharat Biotech, India) and ROTASIIL (Serum Institute of India). Both Rotavac and ROTASIIL are in use only in India, whereas Rotavac is used in India and Palestine. Besides pronounced immune responses against the capsid protein, antibody responses against other rotavirus antigens such as VP6, VP2, NSP2 and NSP4 were also detected. However, the mechanisms by which antiviral immunity is acquired are not clearly understood [13]. Because the introduction of RVA vaccination requires a thorough documentation of disease burden, viral diversity, many countries have initiated RVA surveillance. In Africa, the first countries to introduce RVA vaccination were South Africa (2009) and Morocco (2010) [14]. Rotavirus evolution at the genotypic and sub genotypic levels helps to understand transmission dynamics, where new genotypes emerge through recombination of wild-type and vaccine strains, due to selection pressure (post-vaccine strain shift) [15], or due to gene reassortment [16,17]. Therefore, the question remains how antigenic drift can evade adaptive immunity and thus affect vaccine responses [18,19].

In Gabon, a national immunization program for RVA has not yet been implemented. Furthermore, the information on RVA diversity and burden is poorly documented. Some of the pathogenic RVA genotypes (G1, G2, G3, G12, G6, P[4], P[6], P[8]) were previously detected in four different cities of Gabon, as well as one emerging G6P[6] strain [20]. Thus, pursuing national and regional surveillance is important due to rapid evolution including zoonotic transmission with increased odds of emergence of novel strains. To this end, the aim of the present study is to describe epidemiology and genetic diversity of RVA in Gabonese children <5 years old and to investigate antigenic variability of circulating strains in relation to available vaccines.

2. Methods

2.1. Ethics statement

The study protocol was approved by the Institutional Ethical Committee of the Centre de Recherches Médicales de Lambaréné (CERMEL) (CEI-CERMEL: 003/2017). Written informed consent was obtained from parents or legal representatives of the children.

2.2. Study population and sampling

Between April 2018 and November 2019, stool samples were collected from children under 5 years of age who were residents of semi-urban Lambaréné and its surrounding rural area. The population considered here is representative of the general population because children aged 0–5 years who presented to the outpatient clinics of the two major hospitals in Lambaréné were examined and included if they suffered from diarrhoea (defined as three or more liquid stools within 24 h during the previous three days) and lived in the study area (within a radius of approximately 20 km from Lambaréné). The sample size was calculated based on an estimated rotavirus prevalence among Gabonese children with diarrhoea (27.1%) from a previous [20]. The significance level was 0.05 (corresponding to a 95% confidence interval) with a precision of 0.07 for a sample size of 155 children. Rainy seasons (March-May, October-December) of similar length are interspersed between short (January and February) and long (June–September) dry seasons. Children with diarrhea or history of diarrhea within the last 24 h were recruited at outpatient department of two main hospitals (Hôspital Albert Schweitzer and Centre Hospitalier Régional Georges Rawiri de Lambaréné). In addition, stool samples were randomly collected from healthy children of the same age without gastroenteritis who resided in the same compound as index cases. The collected stool samples were immediately transported to the laboratory and were stored in RNAlater at −20°C for further use.

2.3. Rotavirus detection

The suspension of 200 mg stool in 1 ml of RNAlater solution was homogenized by vortexing and centrifuged. Viral RNA was then extracted from 140 μl of the supernatant with the use of QIAamp viral RNA Mini Kit (QIAGEN, Hilden Germany). All steps of the RNA isolation were performed following the manufacturer's instructions. The genomic RNA was eluted in a total volume of 60 μl, concentration was immediately measured on a Qubit dsRNA XR Assay Kit and the Qubit 4.0 fluorometer (Invitrogen, Paisley, UK) and subsequently stored at − 80°C.

The presence of RVA was detected using a one-step reverse transcription real time PCR (RT-qPCR) protocol for the amplification of the NSP4 gene [21]. Briefly, RNA extracts were first diluted and denaturated at 95 C for 1 min, then RT-qPCR was carried out in a total volume of 12 μl using the SuperScript III/Platinum Taq OneStep kit (Invitrogen, Carlsbad, CA). The reaction mix contained PCR buffer, forward primer (RoA 25-s) 5′GCTTTTAAAAGTT-CTGTTCCGAG), and reverse primer (RoA 26a-as) 5′−ACTCAATGTGTAGTTGAGGTCGG, probe 5′−VIC−ATCTTTCCGCAC-GC−MGB and Platinum Enzyme mix. Samples with a cycle threshold (CT) $\leq$ 39 were considered positive.

2.4. Rotavirus genotyping

For the rotavirus G/P typing we used two RT-semi-nested PCR protocols, specific for each type, as described [22,23]. For the first round, pan-specific primers located close to the 3' and 5' end of the segments were used. In the second run, one of the pan-specific primer was used as a fluorescence labelled primer (with HEX for G types and FAM for P types). The second primer is specific for each P or G Type. This results in fluorescence labelled amplicons with different fragment length. The amplicons generated in the first and second PCRs were also independently checked using agarose gel electrophoresis. For each sample, the fluorescence labelled amplicons generated in the second PCR (nested-PCR round) were mixed with a fluorescence labelled ladder and then analyzed using capillary sequencer. Thus, specific peaks for G and P type could be determined. Additionally, the amplicons generated in the first round of the G/P typing were sequenced using the PCR primer used in the first PCR round. Those samples with a weak signal in the fragment length analysis and/or negative in the first PCR round, a P or G type specific PCR was additionally performed. Some samples showed a signal in the fragment length analysis, but no amplicons were detectable in the first PCR round. For these samples, a VP4- or VP7-long amplification was attempted to identify their genotypes. All amplicons were Sanger sequenced for further analysis. The RVA nucleotide sequences of this study are available under the following GenBank accession numbers: MZ966335 - MZ966498.

2.5. Sequence data analysis

Generated sequences were analyzed in Geneious Prime v2021.1.1 (Biomatters, Auckland, New Zealand). Genotype was assigned using NCBI BLAST and the VIPR typing tool for rotavirus A genotype determination (https://www.viprbrc.org). Nucleotide sequences of VP7 and VP4 were aligned with MAFFT implemented in Geneious and neighbor-joining phylogenetic trees were reconstructed using MEGA 7.0.26 [24] following substitution model using 1000 bootstrap iterations for evaluation of node support. Amino acid sequence similarity was calculated with the p-distance method. Phylogenetic trees were displayed with iTOL [25] and potential N-linked glycosylation sites were screened with NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/).

2.6. Statistical analysis

Differences of RVA prevalence among children with and without diarrhea were tested using the Chi-square or Fischer exact test, with statistical significance set at two-sided p-value <0.05. Associations of RVA infection with potential risk factors were evaluated by conditional logistic regression and a multiple logistic regression model fitted using a stepwise backward procedure. The variables with a p-value ≥0.30 in the univariate analysis were removed sequentially. We adjusted for potential confounders that may influence the occurrence of RVA (e.g., gender, residence). All statistical tests were performed using R version 4.0.2 [26].

2.7. Role of funding source

The project is part of the GZ EI 1044/1-1 AOBJ 630127 grant funded by DFG. The Funders had any role in study design, data collection, data analyses, interpretation, or writing of report.

3. Results

3.1. Study population

A total of 244 children were recruited in the study. Of these 177 (73%) were symptomatic children aged between 0 and 59 months with a median age of 12 months, whereas the median age of healthy children was 24 months. Most participating children (78%; 190/244) were aged 0-24 months. A total of 152 (62%) participants lived in the semi-urban area. Male children were 57% (101/177) in the symptomatic group and 61% (41/67) in the asymptomatic group.

3.2. RVA Prevalence and seasonal distribution

RVA was detected in 98/177 (55%) of symptomatic children and 14/67 (21%) of controls. High RVA detection rate was associated with age, sex, and residence among symptomatic children (Table 1). Children between 0 and 6 months showed the highest proportion of infection (30/47, 64%), while the lowest detection rate was observed among children between 19 and 24 months (5/16, 31%). Females were more often infected (44/76, 58%) than males (54/101, 54%), with similar observations in children from rural (60%) compared to those from semi-urban settings (53%). In the control group, children between 7 and 12 months showed the highest proportion of infections (4/8, 50%). Males were more likely to be infected (10/41, 24%) than females (4/26, 15%), with similar observations in children from rural (31%) compared to those from semi-urban settings (7%).

Gabon, a tropical country has both wet and dry seasons. RVA infection among children with diarrhea was not significantly associated with either dry or wet season (χ^2 = 0.31, p=0.5). However, a significant difference in RVA positive cases was observed between the cumulated long dry and wet seasons (χ^2= 5.06, p=0.02), with peak

Table 1
Rotavirus burden in the study population.

	RVA in Children with acute gastroenteritis cases n (%)	RVA in Children without acute gastroenteritis cases n (%)	p-value
Total	98/177 (55)	14/67 (21)	<0.0001
Age (months)			
0-6	30/47 (64)	0/7 (0)	0.002
7-12	24/47 (51)	4/8 (50)	1
13-18	27/45 (60)	0/4 (0)	0.0345
19-24	5/16 (31)	3/16 (19)	0.6851
25-59	12/22 (55)	7/32 (22)	0.0205
Gender			
Female	44/76 (58)	4/26 (15)	0.0002
Male	54/101 (54)	10/41 (24)	0.0016
Residential area			
Rural	32/53 (60)	12/39 (31)	0.0062
Semi-urban	66/124 (53)	2/28 (7)	<0.0001

RVA infection during September and October 2018 (turn of the seasons) and August-September 2019 (dry season) (Fig. 1a, 1b).

3.3. Genotype distribution and risk factors

In the 112 RT-qPCR positives, we identified two P types and five G types, either in single or mixed infections (Table 2). The P[8] (38.4%) was predominant, followed by P[6] (31%). The most prevalent G type identified was G12 (25%) followed by G1 (18%), G8 (18%), G3 (12%) and G9 (2%). The G type could not be assigned in 31 cases (26%) cases, while the P type identification failed for 34 positive samples (30%) (Supplementary Table S1). The frequent G/P combinations were G12P [6] (21%), G1P[8] (17%) and G8P[8] (15%) (Table 2).

Crude analysis indicated that nutrition, source of drinking water and diarrhea were associated with RVA. Children nourished with formula milk (OR=1.5; 95% CI: 0.55−4.12, p-value= 0.013) and drinking water from wells (OR=1.29; 95%CI: 0.61−2.80; p-value= 0.009) were at higher risk of acquiring a RVA infection. The RVA detection rate was significantly associated with diarrhea (OR=4.63; 95%CI: 2.45−9.29, p-value = <0.001). After adjusting for potential confounders, RVA infection remained significantly associated with diarrhea (OR=3.5; 95%CI: 1.5−8.58, p-value=0.004), and equally for semi-urban population (OR=0.46; 95%CI: 0.21-0.96, p-value= 0.04) (Supplementary Table S2).

3.4. Phylogenetic analysis with vaccine strains

RVA strains belonging to G8, G9, G12, P[6] and P[8] clustered within a single lineage per genotype (Figs. 2, 3). The Gabonese G1 clustered as lineage I and II, showing genetic homogeneity to African and Asian strains, respectively. The Gabonese G1 (lineage II) revealed a higher amino acid pairwise identity with the Rotarix G1 (98%) than the homologous component of RotaTeq. A low pairwise identity was observed between the protein sequence of Gabonese G3 strains and homologous component of RotaTeq (94-95%), as indicated also by their phylogenetic branching. Gabonese G3 strains grouped in lineage III with European, Indian and African strains. Additionally, we have detected the VP7 nucleotide sequence of a G3 strain (GAB/449) closely related to a bat-borne G3P[3] earlier detected in Gabon. The Gabonese G8 clustered in lineage I, showing a close relationship with Eastern Asian RVA (Japan), similarly to the G9 sequences of lineage VI (China and Japan). The G12 sequences formed a relatively homogenous cluster within lineage III, along African, Asian and European strains.

For both P[6] and P[8] genotypes, their VP4 sequences clustered within lineage I and lineage III, respectively. However, the analysis of all symptomatic children revealed a pattern of P type segregation in Gabonese strains, indicative of their association with different G types (Figs. 2, 3). The Gabonese P[8] strains had 93-96% similarity with RotaTeq strain and 90% with the Rotarix P[8]. Furthermore, the P[8] associated with G1 were more similar with RotaTeq and Rotarix (96% and 90%, respectively) than G8P[8] (93-94% and 89-90%, respectively).

3.5. Analysis of VP7 and VP4 neutralizing epitopes

Vaccine efficacy can be undermined if structural differences accumulate in the antigenic epitopes of circulating RVA strains. We used comparative protein analysis to highlight potential antigenic differences between Gabonese RVAs and strains of vaccines that may be introduced. Mutation sites with potential for neutralization escape by monoclonal antibodies reside in the VP7 trimer on two structurally defined antigenic epitopes: 7-1 and 7-2 [27] (Fig. 4). The immunodominant 7-1 epitope is further divided into 7-1a and 7-1b. Overall, the highest number of amino acid differences was observed in the 7-1b subunit of VP7, followed by the 7-2 subunit and the 7-1a subunit. We observed only 5 of 29 amino acid residues completely conserved (W98, Q104, Q201, G264, K291). Most of these sites (W98, Q104, K291) were located on 7-1a. Due to the low viral load and high ct values, we could only obtain only sequence information from only one asymptomatic child.

The VP7 antigenic epitopes of Gabonese G1 strains revealed four residue differences relative to Rotarix G1 and five changes compared to the RotaTeq G1. These sites were found on the epitopes 7-1a and

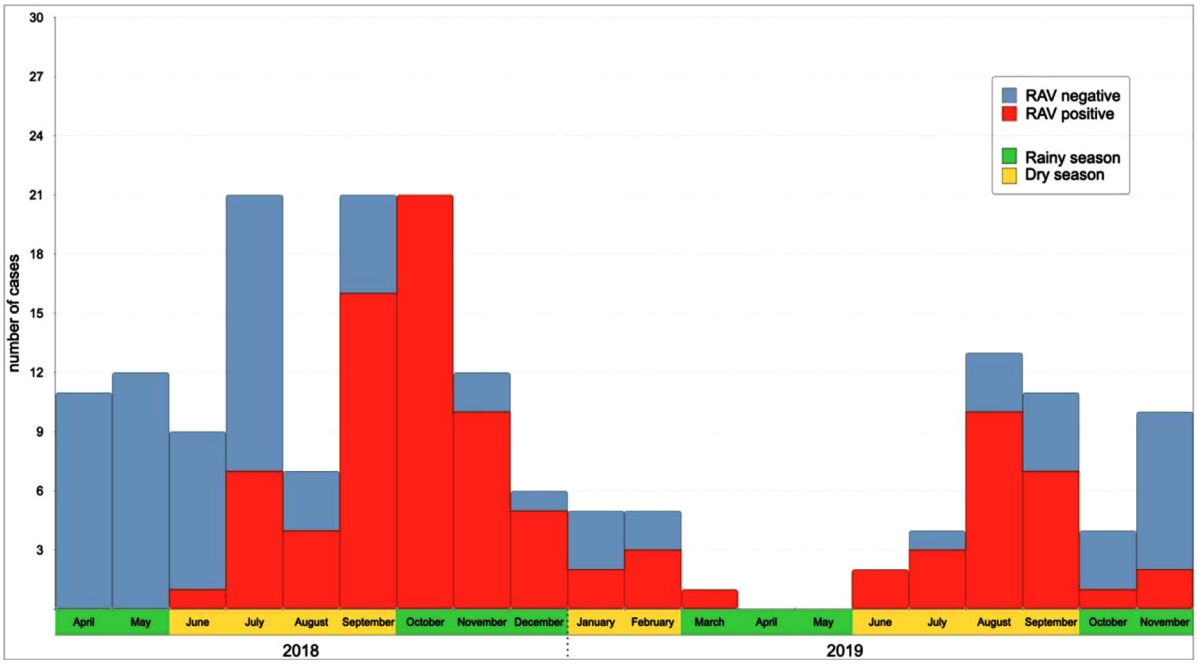

Fig. 1. a. Seasonal burden of rotavirus A among Gabonese children. **b.** Climate graph with recorded temperature and precipitation data of Lambaréné, during the study period (April 2018 until November 2019)

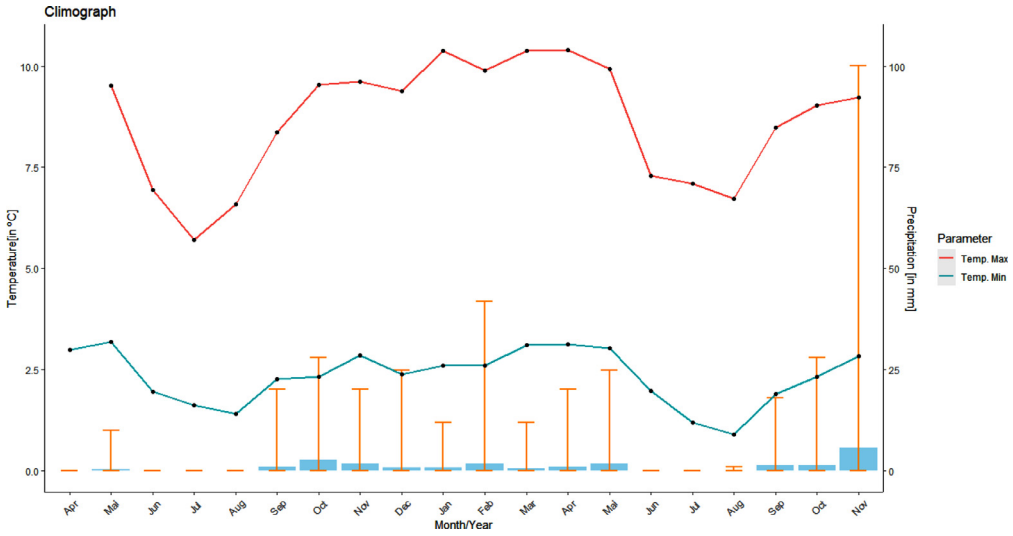

Fig. 1. Continued.

Table 2
Rotavirus genotype (G/P type) distribution in Gabonese children.

	G (X) P types n (%)			
	P [6]	P [8]	P[x]	Total
G1	0	19 (17)	1 (1)	20 (18)
G3	8 (7)	0	1 (1)	9 (8)
G8	0	17 (15)	1 (1)	18 (16)
G9	0	2 (2)	0	2 (2)
G12	24 (21)	2 (2)	0	26 (23)
Gx	1 (1)	1 (1)	29 (21)	31 (28)
Gmix	2 (2)	2 (2)	2 (2)	6 (5)
Total	35 (31)	43 (39)	34 (30)	112 (100)

Mixed types: G1+G3P[8] (1%), G1+G8+G12P[8] (1%), G3+G12P[6] (2%);
Partial G/P mixed types: G3+G8P[x] (1%), G8+G12P[x] (1%).

7-2, respectively. The analysis of G3 showed four to five amino acids different from the RotaTeq G3, distributed on 7-1b and 7-2 epitopes and up to 13 differences relative to Rotarix G1. Several exhibited features of escape mutants [28]. The change in the N94S residue was only observed in strain GAB/675, while the entire antigenic region 7-1b of the G3 strains matches the RotaTeq. All G3 strains carried a mutation at position 238 (epitope 7-1), with most having a substitution (K238N) that modulates N-glycosylation, with one exception in the GAB/449 strain (K238D), which was replaced by aspartic acid. In the 7-2 subunit, mutations at positions 147-148 can modulate responsiveness to specific antibodies; however, only position 147 had amino acid substitutions for both G1 and G3 strains. Most G1 strains had dissimilar residues (N/S147D), while GAB/449 (G3), phylogenetically related to the bat RVA, had a T147A substitution previously associated with immune escape [28,29]. In the VP8* region, the P[8] type revealed distinct signatures at positions N135D, S190N that could well differentiate RotaTeq and Rotarix [30], Whereas the amino acid residues associated with immune escape that differ from both RotaTeq and Rotarix P[8] are located on epitope 8-1 (S146G, N150S), epitope 8-3 (D116N) and epitope 8-4 (N89T) [31].

The comparison of G8, G9 and G12 with human G1-G4 and bovine G6 strains of RotaTeq and the G1 strain of Rotarix showed that Gabonese G8 strains contained four amino acids (sites 96, 145, 147, 213) that are not present in any RotaTeq strain, and 11 residue differences compared to the closest (G3) RotaTeq homolog. In comparison to Rotarix, Gabonese G8 strains contained 15 amino acid differences. Gabonese G9 strains had only two residues changes relative to all RotaTeq strains (sites 94 and 242), each located on the two subunits of the 7-1 epitope, but up to 12 amino acid difference in relation to the closest RotaTeq strain (G3). When compared to the Rotarix strain, the Gabonese G9 showed 14 amino acid changes. G12 strains were the most divergent, having a minimum of 9 different amino acids when compared to VP7 epitopes of vaccine strains. Relative to the closest RotaTeq strain (G3), G12 contained 15 residue changes. The most extensive epitope divergence was observed in relation to Rotarix G1, with 17 residues (Fig. 4).

Following the virion's trypsin activation in the intestine, the VP4 spike protein is proteolytically cleaved in two components: the globular head (VP8*) placed on top of the stalk (VP5*). We analyzed the region coding for the VP8* globular head, the main determinant of RVA P type containing four surface-exposed antigenic epitopes: 8-1, 8-2, 8-3 and 8-4 [31]. Among the Gabonese P[8], P[6] and all vaccine strains we found only two completely conserved sites in epitopes 8-2 (E180) and 8-3 (N132), of the 25 present in VP8* (Fig. 5). The Gabonese P[8] contained up to 24 identical amino acids compared to the P[8] of RotaTeq and 19 identical amino acids relative to P[8] of Rotarix. Six amino acids differences located mostly on epitopes 8-1, 8-3 and 8-4 were shared with both P[8] vaccine strains. The highest number of differences (10 residues) was noted between Gabonese G1P[8] strain GAB/675 and Rotarix P[8].

The epitope composition of the Gabonese P[6] strains relative to vaccine VP8* showed extensive dissimilarity, consistent with the levels of sequences divergence. P[6] strains associated with G3 contained 18-20 residue differences, whilst the G12P[6] contained 17-19 different amino acids.

4. Discussion

In preparation for introducing rotavirus vaccine by the EPI for Gabonese children, we investigated the prevalence and genetic characteristics of RVA among children with and without diarrhea over a 20-month period. The RVA detection rate in diarrheal cases is twice as high as previously reported in Gabon [20]. RVA was present to a greater extent in children with diarrhea than in asymptomatic ones, with similar or higher detection rate than in West/East Africa and South Asia [32,33].

As observed in various studies, high infection was observed in most children aged 0–24 months [33,34]. The significant association of RVA infection with the semi-urban environment could be related to population density and contact rates, but also to living conditions.

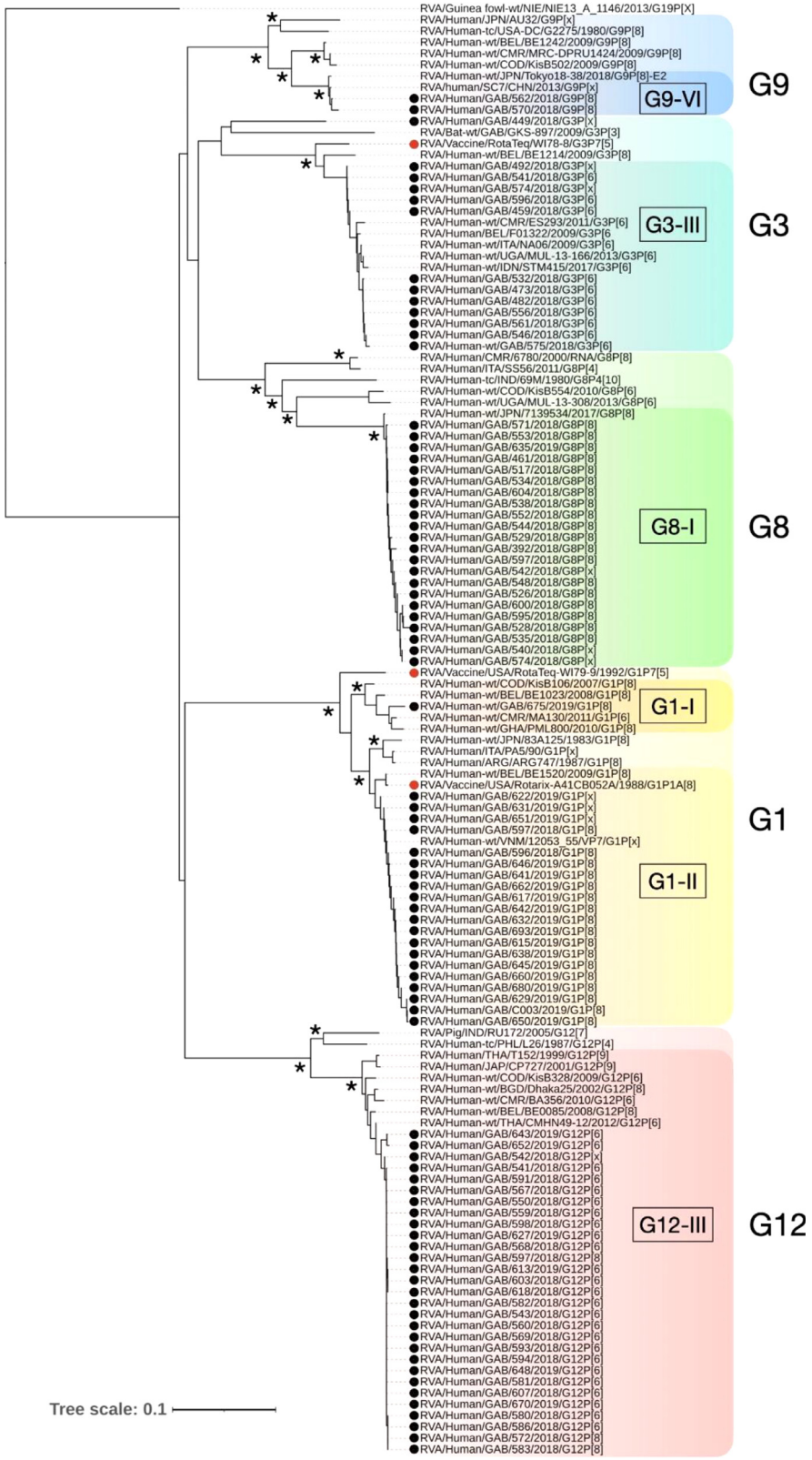

Fig. 2. Phylogenetic analysis of representative RVA strains based on a VP7 gene fragment (856 nucleotides). Gabonese strains analyzed are marked by black dots and vaccine strains are marked by red dots. Guineafowl RVA strain NIE13A1146 was used as an outgroup and bootstrap support >80% is indicated by asterisk. Tree scale bar represents number of substitutions per site.

The AOR analysis showed no significant association with pit toilet use and RVA infections, this toilet type was present in about 90% of RVA positive households, in contrast to flush toilets (9%). The use of public taps as a source of drinking water was reported for more than half (55%) of the infected children, as opposed to water from the household tap (13%). Therefore, such factors may discretely contribute to the acquisition of RVA infections, especially in semi-urban areas with high population density. Over the sampling period, the proportion of

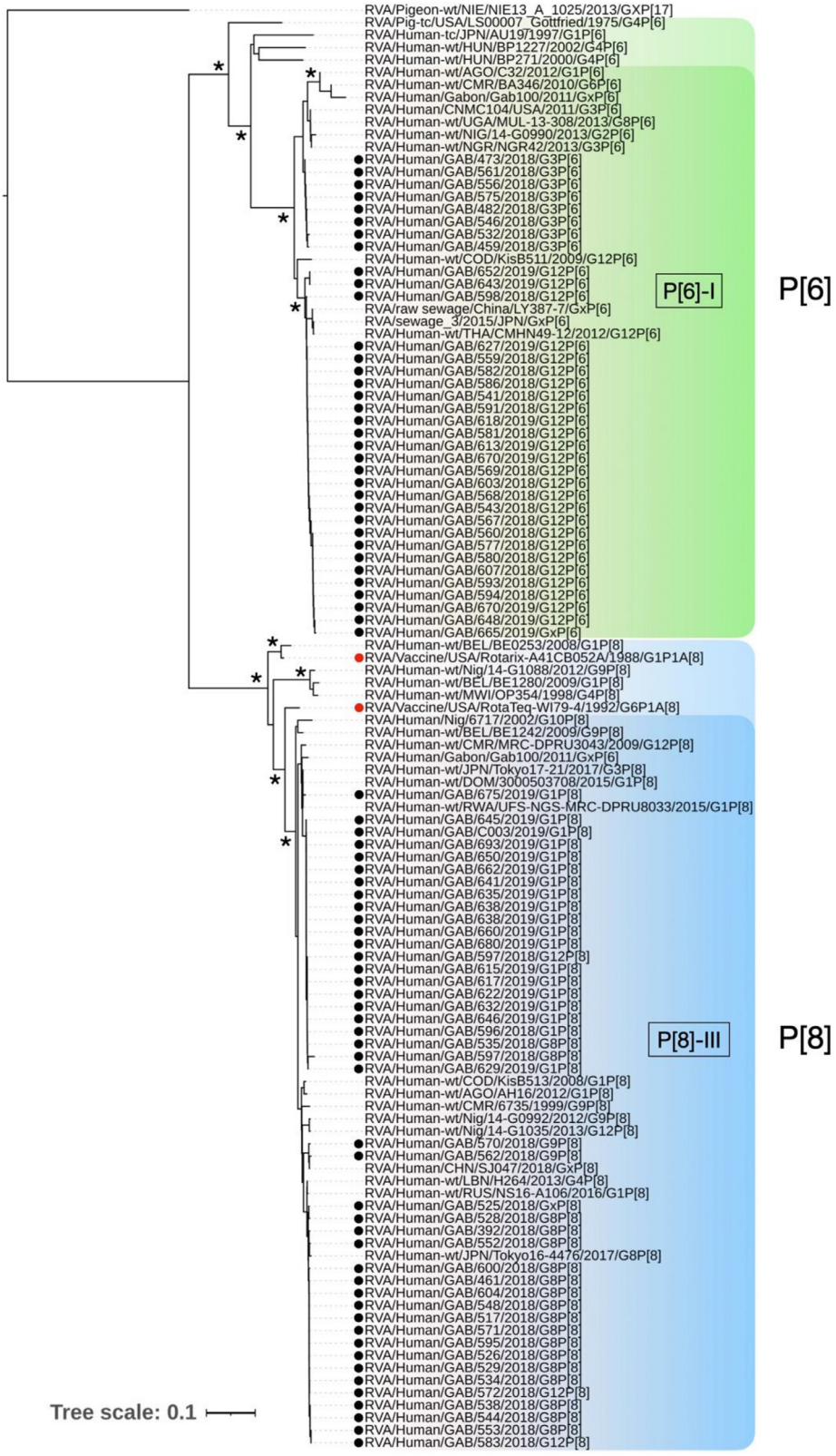

Fig. 3. Phylogenetic analysis of representative RVA strains based on a fragment of VP4, containing the VP8* sequence (645 nucleotides). Gabonese strains analyzed are marked by black dots and vaccine strains are marked by red dots. Pigeon RVA strain NIE13A1025 was used as an outgroup and bootstrap support >80% is indicated by asterisk. Tree scale bar represents number of substitutions per site.

infections differed statistically only between rainy seasons and cumulative long dry seasons. RVA infections peaked in September-October (2018) or August-September (2019), a pattern consistent with previous observations in Gabon [20], West Africa [35,36], Central Africa [37], Southeast Asia [38] and generally in the tropics [39].

The frequencies of G1 and G3 genotypes in the present study population are higher than previously reported in the country [20] and in

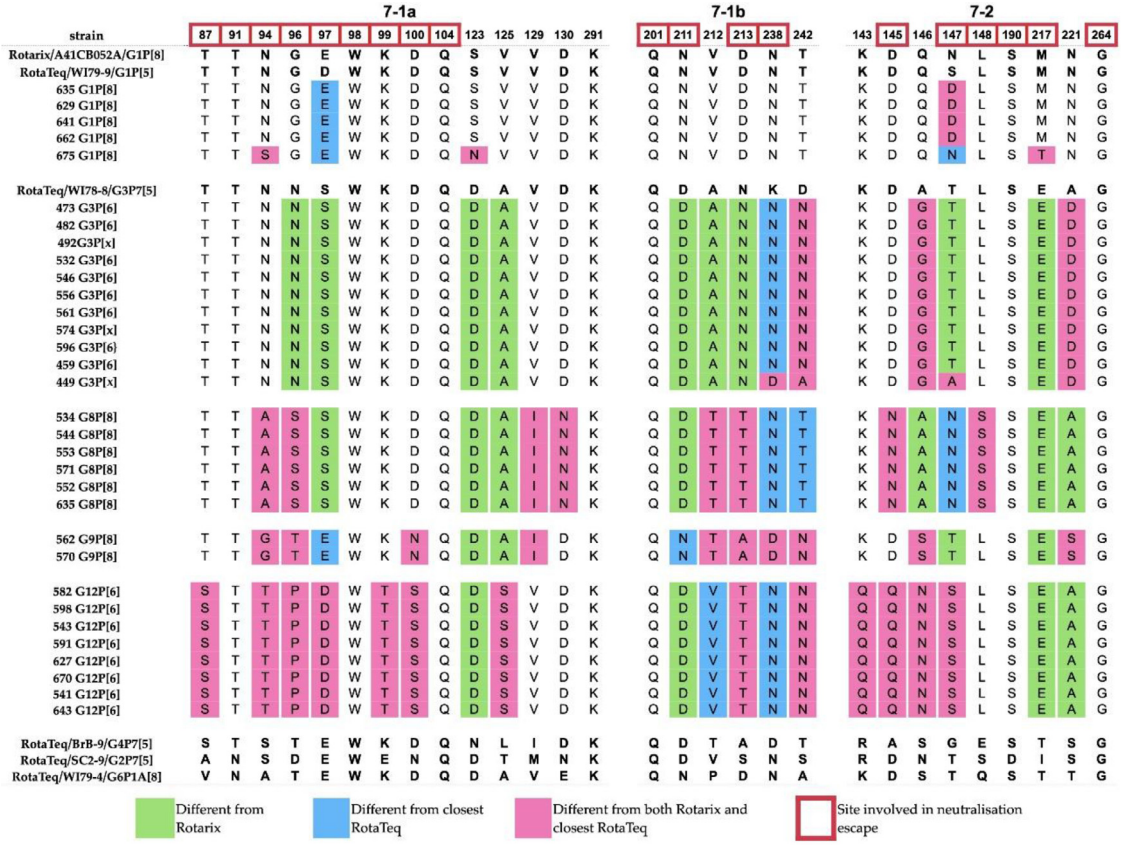

Fig. 4. Comparison of VP7 antigenic epitopes of Gabonese and vaccine strains. Amino acids of vaccine strains are indicated in bold.

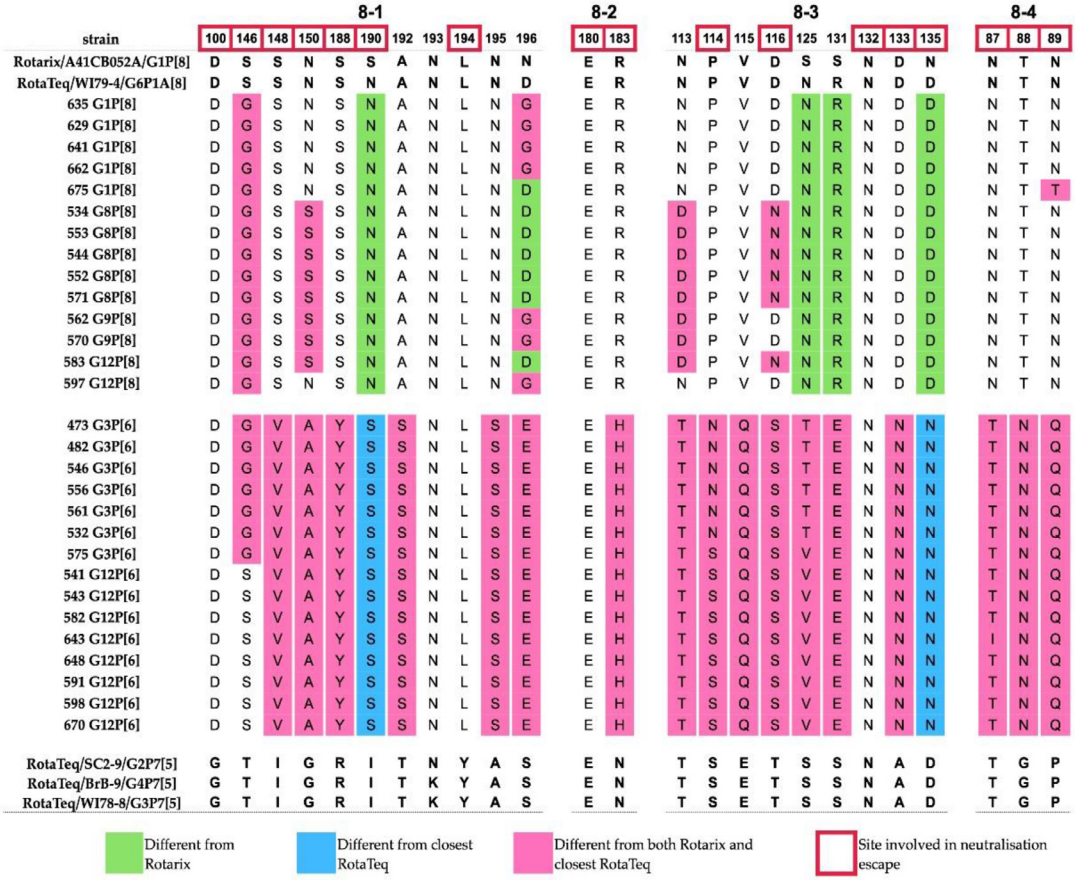

Fig. 5. Comparison of VP4 (VP8*) antigenic epitopes of Gabonese and vaccine strains. Amino acids of vaccine strains are indicated in bold.

neighboring Cameroon [40], while G8 and G9 were detected for the first time in Gabon. In contrast to other Central African countries where G1 is dominant, this genotype was the second most common in our study (18%), while the dominant G12 (25%) was found in similar proportions [34,38]. Although G12 was found at a lower frequency in this study than in Cameroon (67.4%) [40], previous work by Lekana-Douki et al. [20] in Gabon also showed that G12 was less common (11.8%). In contrast to previous work by Lekana-Douki et al. [20], we observed P[8] to be the most frequent P genotype (38%). The other P genotype in our study is P[6] (31%), had a lower detection rate than those previously reported in the country (71.4%). This high incidence of P[8] has been observed not only in Central African countries, but also in Central and South-eastern Europe [41,42]. We observed a high proportion of G1P[8] (17%), although it was significantly lower than in neighboring country (Republic of Congo, 44%) [37]. G12P[8] and G12P[6] were found at lower frequencies compared to Cameroon [40]. In addition, we found G9P[8] and G8P[8], genotypes frequently detected in Southeast Asia [38]. These genotypes considered rare or uncommon, were also reported with low frequencies in other African countries [36]. 28 % of non-typeable types (Gx-Px) were observed and there were many non-typeable types among the P-types. All qPCR-positive samples that were negative in semi-nested RT- PCR were designated as non-typeable. There are two likely reasons that have been raised as limitations of this study. One probable reason is that qPCR has a high sensitivity compared to RT-PCR; this could well be observed in non-typeable samples that gave high ct values in the independently performed tests. The other reason could be due to mutations in the primer binding sites, which impairs annealing. However, the latter explanation is unlikely.

RVA zoonotic genotypes such as G8 (bovine), P[11] (bovine) and P[6] (porcine) are widespread in developing countries and cause infections in humans [43–45]. High diversity of RVA (including untypeable strains) in human populations, livestock or wildlife [46–48] are common. In our study, a G3 strain (GAB/449) obtained from a symptomatic child was phylogenetically closer to a Gabonese strain earlier discovered in a giant round bat (H. gigas, GenBank no. MN528121). Poor sanitary conditions and high contact rates with livestock and wildlife may contribute to spill-over-spillback transmission patterns. Consequently, the rate of infection is also higher (~20%) compared to middle- and high-income countries (~5%) [43].

The phylogenetic analysis of Gabonese G types revealed well supported terminal clades indicating close relatedness with strains of Asian origin. Although P[8] is widespread in Africa, we cannot rule out the possibility of cross-border transmission in Gabon due to the constant influx of migrants from Asia and other countries. The phylogenetic sub-lineage segregation of Gabonese VP4 in discrete clusters following a pattern of G type association (e.g., P[6] with G3 and G12, P[8] with G1 and G8) might be a result of reassortment.

Strains of P[8] contained amino acid disparities in their VP8* epitopes in accordance with their G1, G8 and G9 combinations, although with notable exceptions. G1P[8] was one of the frequent type combinations and most divergent from vaccine cognates, similarly to the less frequent G9P[8]. These genetic and antigenic differences should be considered when planning immunization, since the chosen vaccines will shape the antigenic landscape for circulating RVA. The antigenic G1-VP7 epitopes were mostly conserved and homogeneous with the vaccine strains. Of all three VP7 epitopes, the RVA genome was apparently conserved with vaccine strains, with an exception in G1P[8] (GAB/675), which revealed the N94S substitution associated with immune escape [28,49]. The VP7 epitopes of the Gabonese G3 strains had a relatively low number of different residues compared to RotaTeq G3. Despite this observation, the presence of K238N (epitope 7-1b) may be of concern as it affects glycosylation and has been shown to neutralize RVA in mammals by monoclonal antibodies and hyperimmune sera [46,50]. K238N has also been reported previously in European and North African G3 strains [10,51], with additional sites (70-72) contributing to N-linked glycosylation.

In the case of RVA immunization, a vaccine's failure to eradicate the virus can result in selective pressures that enhance the pathogen's ability to evade host immunity [47]. Viral evolution is often linked to travel and domesticated livestock populations in the community that facilitate the virus shedding and recombination of RVA strains in antigenically naive hosts [48,52]. Although many studies have examined RVA burden in symptomatic patients, this study investigated children without gastroenteritis. The comparison of the RVA genotype distribution between symptomatic and asymptomatic children would have been useful. However, due to the low viral load, we could only obtain a sequence from the control group (asymptomatic children). Another limitation of our study is the small size of the asymptomatic group. This is because sampling began in the second year of the study and therefore precludes observation of the seasonal distribution of RVA cases among asymptomatic children.

The presence of the main pathogenic strains in Gabon, namely G1 and G3 in combination with P[6], the emerging G12 and the less common G9 with antigenic divergence, makes the implementation of vaccination necessary and urgent. This study provides the genetic data needed to assess the epidemiological situation prior to the introduction of vaccination in the Gabonese population, where a wide variety of RVA are present, including strains that are likely to be of zoonotic origin. Additional longitudinal studies at temporal and spatial scales are needed to determine the functional significance of these genetic divergences observed in Gabonese RVA strains and to establish a link with available vaccines, proven to be efficacious.

Contributors

AAA designed, supervised, and coordinated the study procedures. GPM performed all experimental procedures and drafted the manuscript. AT supported experimental procedures, analyzed genetic data, and drafted the manuscript. GPM, MMN, MNM, PANM, GBM, SA and EGR were involved in sampling procedures and collating the baseline data. SN and CTB performed RVA genotyping for independent verification in another laboratory and supported with data analysis. SB, BM, DE, and PGK contributed to the study design and patient recruitment. TPV and AAA contributed to the materials. TPV supervised experimental procedures on RVA screening and genotyping, data analysis, and revised the manuscript from the first draft. All authors read and approved the final version of the manuscript. TPV, AAA, GPM, SN, AT verified the underlying data in this manuscript.

STROBE Statement—Checklist of items that should be included in reports of ***case-control studies***

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract Page 2 – Lines 40–66 (*b*) Provide in the abstract an informative and balanced summary of what was done and what was found Page 2 – Lines 40–66
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported Pages 4 and 5 Lines 112–161
Objectives	3	State specific objectives, including any prespecified hypotheses Pages 4 and 5 Lines 134–161
Methods		
Study design	4	Present key elements of study design early in the paper Page 5, 169–186
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection Page 5, 169–186
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls Page 5, 169–186 (*b*) For matched studies, give matching criteria and the number of controls per case Page 5, 169–186
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable Supplementary data Table S2
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group Page 5, 169–186, (cases and controls) Supplementary data Table S2 (cases)
Bias	9	Describe any efforts to address potential sources of bias Not applicable
Study size	10	Explain how the study size was arrived at Page 5, 169–186,
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why Page 7, 235–242,
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding Page 7, 235–242, (*b*) Describe any methods used to examine subgroups and interactions Page 7, 224–233, (*c*) Explain how missing data were addressed Not applicable (*d*) If applicable, explain how matching of cases and controls was addressed (*e*) Describe any sensitivity analyses
Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed Page 5, 169–186, Page 9, 10, 256–289 (b) Give reasons for non-participation at each stage Not applicable (c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders Page 5, 169–186, (controls and cases) and Supplementary Table S2, (Cases) (b) Indicate number of participants with missing data for each variable of interest Supplementary Table S2
Outcome data	15*	Report numbers in each exposure category, or summary measures of exposure Page 9, 10, 256–289 (controls) and Supplementary Table S2 (cases)
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included Page 9, 10, 256–289 and Supplementary Table S2 (*b*) Report category boundaries when continuous variables were categorized Supplementary Table S2 (*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period Page 9, 10 256–289
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses Page 8–10, 248–371
Discussion		
Key results	18	Summarise key results with reference to study objectives Page 10- 371–379
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias Page 13, 450–469
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence Pages 11–13, 373–469

(*continued*)

(*Continued*)

	Item No	Recommendation
Generalisability	21	Discuss the generalisability (external validity) of the study results Pages 11–13, 373–469
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based Page 7, 244–246

*Give information separately for cases and controls.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

Data sharing statement

All data relevant to the study are included in the article or uploaded as supplementary information.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2021.103648.

References

[1] Troeger C, Forouzanfar M, Rao PC, Khalil I, Brown A, Reiner RC, et al. Estimates of global, regional, and national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the global burden of disease study 2015. Lancet Infect Dis 2017;17(9):909–48.
[2] Crawford SE, Ramani S, Tate JE, Parashar UD, Svensson L, Hagbom M, et al. Rotavirus infection. JAMA Pediatr 2018;172(SUPPL. 3):50–3 [Internet]Available from http://repositorio.uchile.cl/bitstream/handle/2250/148901/Rotavirus-infection.pdf?sequence=1&isAllowed=y.
[3] Matthijnssens. Uniformity of rotavirus strain nomenclature proposed by the rotavirus classification working group (RCWG) jelle. Arch Virol 2011;156(8):1397–413.
[4] Matthijnssens J, Ciarlet M, Rahman M, Attoui H, Estes MK, Gentsch JR, et al. Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments. Arch Virol 2008;153(8):1621–9.
[5] Azaran A, Makvandi M, Teimoori A, Ebrahimi S, Heydari F, Nikfar R. Distribution of rotavirus genotypes circulating in Ahvaz, Iran in 2016. Iran Biomed J 2018;22(2):107–16.
[6] Santos N, Hoshino Y. Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine. Rev Med Virol 2005;15(1):29–56.
[7] Todd S, Page NA, Steele AD, Peenze I, Cunliffe NA. Rotavirus strain types circulating in africa: Review of studies published during 1997–2006. J Infect Dis 2010;202(SUPPL. 1):34–42.
[8] Doro R, Farkas SL, Martella V, Banyai K. Zoonotic transmission of rotavirus: surveillance and control. Expert Rev Anti Infect Ther 2015;13(11):1337–50.
[9] Gentsch JR, Laird AR, Bielfelt B, Griffin DD, Bányai K, Ramachandran M, et al. Serotype diversity and reassortment between human and animal rotavirus strains: Implications for rotavirus vaccine programs. J Infect Dis. 2005;192(SUPPL. 1):46–59.
[10] Zeller M, Patton JT, Heylen E, De Coster S, Ciarlet M, Van Ranst M, et al. Genetic analyses reveal differences in the VP7 and VP4 antigenic epitopes between human rotaviruses circulating in Belgium and rotaviruses in rotarix and RotaTeq. J Clin Microbiol 2012;50(3):966–76.
[11] Bányai K, László B, Duque J, Steele AD, Nelson EAS, Gentsch JR, et al. Systematic review of regional and temporal trends in global rotavirus strain diversity in the pre rotavirus vaccine era: Insights for understanding the impact of rotavirus vaccination programs. Vaccine 2012;30(SUPPL. 1):122–30.
[12] Burke RM, Tate JE, Kirkwood CD, Steele AD, Parashar UD. Current and new rotavirus vaccines. Curr Opin Infect Dis 2019;32(5):435–44.
[13] Ward RL, Clark HF, Offit PA. Influence of potential protective mechanisms on the development of live rotavirus vaccines. J Infect Dis 2010;202(SUPPL. 1):72–9.
[14] Mwenda JM, Tate JE, Steele AD, Parashar UD. Preparing for the scale-up of rotavirus vaccine introduction in Africa: establishing surveillance platforms to monitor disease burden and vaccine impact. Pediatr Infect Dis J 2014;33(SUPPL. 1):1–5.
[15] Roczo-Farkas S, Kirkwood CD, Cowley D, Barnes GL, Bishop RF, Bogdanovic-Sakran N, et al. The impact of rotavirus vaccines on genotype diversity: a comprehensive analysis of 2 decades of australian surveillance data. J Infect Dis 2018;218(4):546–54.
[16] Bucardo F, Rippinger CM, Svensson L, Patton JT. Vaccine-derived NSP2 segment in rotaviruses from vaccinated children with gastroenteritis in Nicaragua. Infect Genet Evol 2012;12(6):1282–94.
[17] Rose TL, da Silva MFM, Goméz MM, Resque HR, Ichihara MYT, Volotão E de M, et al. Evidence of vaccine-related reassortment of rotavirus, Brazil, 2008–2010. Emerg Infect Dis 2013;19(11):1843–6.
[18] Diwakarla CS, Palombo EA. Genetic and antigenic variation of capsid protein VP7 of serotype G1 human rotavirus isolates. J Gen Virol 1999;80(2):341–4.
[19] Matthijnssens J, Bilcke J, Ciarlet M, Martella V, Bányai K, Rahman M, et al. Rotavirus disease and vaccination: impact on genotype diversity. Future Microbiol. 2009;4(10):1303–16.
[20] Lekana-Douki SE, Kombila-Koumavor C, Nkoghe D, Drosten C, Drexler JF, Leroy EM. Molecular epidemiology of enteric viruses and genotyping of rotavirus A, adenovirus and astrovirus among children under 5 years old in Gabon. Int J Infect Dis 2015;34:90–5.
[21] Japhet MO, Famurewa O, Iturriza-Gomara M, Adesina OA, Opaleye OO, Niendorf S, et al. Group A rotaviruses circulating prior to a national immunization programme in Nigeria: clinical manifestations, high G12P[8]frequency, intra-genotypic divergence of VP4 and VP7. J Med Virol 2018;90(2):239–49.
[22] Iturriza-Gómara M, Kang G, Gray J. Rotavirus genotyping: Keeping up with an evolving population of human rotaviruses. J Clin Virol 2004;31(4):259–65.
[23] Marques AM, Diedrich S, Huth C, Schreier E. Group A rotavirus genotypes in Germany during 2005/2006. Arch Virol 2007;152(9):1743–9.
[24] Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 2018;35(6):1547–9.
[25] Letunic I, Bork P. Interactive tree of life (iTOL) v4: recent updates and new developments. Nucleic Acids Res 2019;47(W1):W256–9.
[26] R. Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/. 2020.
[27] Aoki ST, Settembre E, Trask SD, Greenberg HB, Stephen C, Dormitzer PR. Structure of rotavirus outer-layer protein VP7 bound with a neutralizing Fab. Science 2009;324(5933):1444–7 80-.
[28] Coulson BS, Kirkwood C. Relation of VP7 amino acid sequence to monoclonal antibody neutralization of rotavirus and rotavirus monotype. J Virol 1991;65(11):5968–74.
[29] Dyall-Smith ML, Lazdins I, Tregear GW, Holmes IH. Location of the major antigenic sites involved in rotavirus serotype-specific neutralization. Proc Natl Acad Sci USA 1986;83(10):3465–8.
[30] Giammarioli AM, Mackow ER, Fiore L, Greenberg HB, Ruggeri FM. Production and characterization of murine IgA monoclonal antibodies to the surface antigens of rhesus rotavirus. Virology 1996;225(1):97–110.
[31] Dormitzer PR, Sun ZYJ, Wagner G, Harrison SC. The rhesus rotavirus VP4 sialic acid binding domain has a galectin fold with a novel carbohydrate binding site. EMBO J 2002;21(5):885–97.
[32] Odimayo MS, Olanrewaju WI, Omilabu SA, Adegboro B. Prevalence of rotavirus-induced diarrhoea among children under 5 years in Ilorin, Nigeria. J Trop Pediatr 2008;54(5):343–6.
[33] Iturriza-Gómara M, Jere KC, Hungerford D, Bar-Zeev N, Shioda K, Kanjerwa O, et al. Etiology of diarrhea among hospitalized children in blantyre, malawi, following rotavirus vaccine introduction: a case-control study. J Infect Dis 2019;220(2):213–8.

[34] Shrestha S, Thakali O, Raya S, Shrestha L, Parajuli K, Sherchand JB. Acute gastroenteritis associated with rotavirus A among children less than 5 years of age in Nepal. BMC Infect Dis 2019;19(1):1–8.
[35] Ouedraogo N, Ngangas SMT, Bonkoungou IJO, Tiendrebeogo AB, Traore KA, Sanou I, et al. Temporal distribution of gastroenteritis viruses in Ouagadougou, Burkina Faso: seasonality of rotavirus. BMC Public Health 2017;17(1):1–8.
[36] Mwenda JM, Ntoto KM, Abebe A, Enweronu-Laryea C, Amina I, Mchomvu J, et al. Burden and epidemiology of rotavirus diarrhea in selected african countries: Preliminary results from the african rotavirus surveillance network. J Infect Dis 2010;202(SUPPL. 1):5–11.
[37] Mayindou G, Ngokana B, Sidibé A, Moundélé V, Koukouikila-Koussounda F, Christevy Vouvoungui J, et al. Molecular epidemiology and surveillance of circulating rotavirus and adenovirus in Congolese children with gastroenteritis. J Med Virol 2016;88(4):596–605.
[38] Lestari FB, Vongpunsawad S, Wanlapakorn N, Poovorawan Y. Rotavirus infection in children in Southeast Asia 2008-2018: disease burden, genotype distribution, seasonality, and vaccination. J Biomed Sci 2020;27(1):1–19.
[39] Levy K, Hubbard AE, Eisenberg JNS. Seasonality of rotavirus disease in the tropics: a systematic review and meta-analysis. Int J Epidemiol 2008;38(6):1487–96.
[40] Ndze VN, Papp H, Achidi EA, Gonsu KH, László B, Farkas S, et al. One year survey of human rotavirus strains suggests the emergence of genotype G12 in Cameroon. J Med Virol 2013;85(8):1485–90.
[41] Moure UAE, Banga-Mingo V, Gody JC, Mwenda JM, Fandema J, Waku-Kouomou D, et al. Emergence of G12 and G9 rotavirus genotypes in the Central African Republic, January 2014 to February 2016. BMC Res Notes 2018;11(1):5. [Internet]Available from. doi: 10.1186/s13104-017-3122-7.
[42] Tcheremenskaia O, Marucci G, De Petris S, Ruggeri FM, Dovecar D, Sternak SL, et al. Molecular epidemiology of rotavirus in Central and Southeastern Europe. J Clin Microbiol 2007;45(7):2197–204.
[43] Iturriza Gómara M, Kang G, Mammen A, Jana AK, Abraham M, Desselberger U, et al. Characterization of G10P[11]rotaviruses causing acute gastroenteritis in neonates and infants in Vellore, India. J Clin Microbiol 2004;42(6):2541–7.
[44] Malasao R, Khamrin P, Kumthip K, Ushijima H, Maneekarn N. Complete genome sequence analysis of rare G4P[6]rotavirus strains from human and pig reveals the evidence for interspecies transmission. Infect Genet Evol 2018;65:357–68.
[45] Matthijnssens J, Ciarlet M, Heiman E, Arijs I, Delbeke T, McDonald SM, et al. Full genome-based classification of rotaviruses reveals a common origin between human wa-like and porcine rotavirus strains and human DS-1-like and bovine rotavirus strains. J Virol 2008;82(7):3204–19.
[46] Ciarlet M, Hoshino Y, Liprandi F. Single point mutations may affect the serotype reactivity of serotype G11 porcine rotavirus strains: a widening spectrum? J Virol 1997;71(11):8213–20.
[47] Read AF, Baigent SJ, Powers C, Kgosana LB, Blackwell L, Smith LP, et al. Imperfect vaccination can enhance the transmission of highly virulent pathogens. PLoS Biol 2015;13(7):1–18.
[48] Pybus OG, Tatem AJ, Lemey P. Virus evolution and transmission in an ever more connected world. Proc R Soc B Biol Sci 2015;282(1821):1–10.
[49] Taniguchi K, Hoshino Y, Nishikawa K, Green KY, Maloy WL, Morita Y, et al. Cross-reactive and serotype-specific neutralization epitopes on VP7 of human rotavirus: nucleotide sequence analysis of antigenic mutants selected with monoclonal antibodies. J Virol 1988;62(6):1870–4.
[50] Ciarlet M, Reggeti F, Pina CI, Liprandi F. Equine rotaviruses with G14 serotype specificity circulate among Venezuelan horses. J Clin Microbiol 1994;32(10):2609–12.
[51] Mouna BHF, BenHamida-Rebaï M, Heylen E, Zeller M, Moussa A, Kacem S, et al. Sequence and phylogenetic analyses of human rotavirus strains: comparison of VP7 and VP8* antigenic epitopes between Tunisian and vaccine strains before national rotavirus vaccine introduction. Infect Genet Evol 2013;18(May):132–44 [Internet]Available from. doi: 10.1016/j.meegid.2013.05.008.
[52] Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, et al. Global trends in emerging infectious diseases. Nature 2008;451(7181):990–3.

2.4. Chapter 4

Genetic diversity of enteric viruses in children under five years old in Gabon

Manouana GP, Nguema-Moure PA, Mbong Ngwese M, Bock CT, Kremsner PG, Borrmann S, Eibach D, Mordmüller B, Velavan TP, Niendorf S, Adegnika AA.

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Genetic Diversity of Enteric Viruses in Children under Five Years Old in Gabon

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Abstract: Enteric viruses are the leading cause of diarrhea in children globally. Identifying viral agents and understanding their genetic diversity could help to develop effective preventive measures. This study aimed to determine the detection rate and genetic diversity of four enteric viruses in Gabonese children aged below five years. Stool samples from children <5 years with (n = 177) and without (n = 67) diarrhea were collected from April 2018 to November 2019. Norovirus, astrovirus, sapovirus, and aichivirus A were identified using PCR techniques followed by sequencing and phylogenetic analyses. At least one viral agent was identified in 23.2% and 14.9% of the symptomatic and asymptomatic participants, respectively. Norovirus (14.7%) and astrovirus (7.3%) were the most prevalent in children with diarrhea, whereas in the healthy group norovirus (9%) followed by the first reported aichivirus A in Gabon (6%) were predominant. The predominant norovirus genogroup was GII, consisting mostly of genotype GII.P31-GII.4 Sydney. Phylogenetic analysis of the 3CD region of the aichivirus A genome revealed the presence of two genotypes (A and C) in the study cohort. Astrovirus and sapovirus showed a high diversity, with five different astrovirus genotypes and four sapovirus genotypes, respectively. Our findings give new insights into the circulation and genetic diversity of enteric viruses in Gabonese children.

Keywords: enteric viruses; children; phylogenetic analysis; diarrhea; Gabon

1. Introduction

Gastroenteritis (GE) remains a major public health issue worldwide, especially among children [1]. Globally, more than 700 million cases of acute gastroenteritis in children below 5 years, and 0.8–2 million deaths, per year, have been estimated [2]. GE can have several causes. Previous studies have identified rotavirus, norovirus, astrovirus, and adenovirus as major viral etiologies of diarrheal illness [3–6]. In particular, rotavirus group A (RVA) (family Reoviridae) remains the most important etiological agent of severe diarrhea in children under five years of age, with an estimated 215,000 deaths recorded

in 2013 [7,8]. In recent decades, data on the epidemiology and disease burden of RVA infection have contributed to the implementation of RVA vaccination (the monovalent Rotarix (GlaxoSmithKline Biologicals, Rixensart, Belgium) and the pentavalent human-bovine reassortant RotaTeq (Merck, Kenilworth, NJ, USA)). Although recommended by the World Health Organization (WHO), these two RVA vaccines have demonstrated a low efficacy in resource-poor countries [9].

Noroviruses (NoVs) are associated with estimated deaths of over 200,000 annually, with an important proportion occurring in children from developing countries [10,11]. NoVs comprise the *Norovirus* genus within the Calciviridae family and are small, round structured viruses, non-enveloped with a positive-sense single-stranded RNA genome of around 7.5 kb consisting of three open reading frames (ORFs). ORF1 encodes six non-structural proteins including the viral polymerase. ORF2 and ORF3 encode the major and minor capsid proteins VP1 and VP2, respectively [12,13]. NoV strains are segregated into ten genogroups (G), of which genogroups GI, GII, GIV, GVIII, and GIX contain primarily human viruses linked with GE. Through phylogenetic analyses, at least forty-eight genotypes infecting humans were identified, with the GII.4 genotype responsible for at least 70% of infections worldwide [14,15].

Other viral agents, including astroviruses (AstV, family Astroviridae), sapoviruses (SaV, family Caliviridae), and aichiviruses (AiV, family Picornaviridae), are present overall in much smaller proportions than NoVs and RVA. AstVs, single-stranded RNA viruses, are an important cause of diarrhea in children. They are classified into ten genotypes, of which eight are "classic" genotypes (HAstV1–HAstV8), and two have been described only recently (HAstV-MBL and HAstV-VA/HOM) [16,17]. HAstVs have been identified worldwide and the overall detection rate among children with acute gastroenteritis ranges between 0% and 20% [18]. Human SaVs were detected in people of all ages in both outbreaks and sporadic cases worldwide, with a median detection rate of 6.2% (with a range from 0.2% to 39%) among children <5 years old in low- and middle-income countries [19]. Human SaVs are classified to seventeen different genotypes belonging to four genogroups (GI, GII, GIV, and GV) [20]. AiVs were suggested to play a role in GE, especially in outbreaks caused by contaminated seafood [21]. Human pathogenic AiVs belonging to the genus *Kobuvirus* were classified as AiV-1 and are divided into three different genotypes (A-C) [22], with little information available about their geographical distribution.

In Sub-Saharan Africa and South-East Asia, where over 90% of diarrhea-related deaths occur among children younger than 5 years [23], routine diagnostic or surveillance for viral etiology of diarrheal diseases is lacking or limited [24]. A previous epidemiological study conducted in 2015 in four cities of Gabon reported the predominant proportion of RVA, followed by human adenovirus (HAdV), NoVs, SaV, and AstV among symptomatic children [25]. However, following up these results as part of a national surveillance program prior to rotavirus vaccination in Gabon is essential to establish a solid evidence base.

In this study, we assessed the genetic diversity of enteric viruses in Gabonese children younger than 5 years.

2. Materials and Methods

2.1. Sample Collection

In this study, stool samples were collected from children under 5 years of age who resided in one of Gabon's main cities (Lambaréné) and its surroundings (a dominantly rural area without running water and electricity, and reduced access to the health system) between April 2018 and November 2019. Cases were children presenting at the outpatient departments of the two main hospitals with diarrhea or history of diarrhea within the last 24 h. Subsequently, stool samples were randomly obtained from healthy children living in the same neighborhoods and having the same life conditions (source of drinking water, type of toilet, feeding practice, and material of living house) as those of diarrhea cases. All controls were gender- and age-matched with children with diarrhea. The biological

material was immediately transported to the CERMEL laboratory and stored in RNAlater at −20 °C until analysis.

2.2. Viral RNA Extraction

RNA was extracted from 140 µL of the stool suspension in RNAlater spiked with an internal extraction- and PCR-control (MS-2 phage) using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany). All steps of the RNA isolation were performed following the manufacturer's instructions and the viral RNA was eluted in a total volume of 60 µL and stored in aliquots at −70 °C.

2.3. PCR Detection and Genotyping

The presence of NoV and SaV was first screened using a single-step reverse transcription real time PCR as described previously [26,27]. In short, 10 µL mastermix containing Superscript™ III Platinum OneStep RT-qPCR System (Invitrogen, Karlsruhe, Germany) and 2 µL virus RNA extracted was used for the detection. All NoV positive samples were subsequently genotyped based on amplification and nucleotide sequencing of the RNA-dependent RNA polymerase gene (RdRp, ORF1) and the capsid gene (ORF2, P2 region) as previously described [28]. SaV positive samples by RT-qPCR were characterized by amplifying a polymerase region of 650 bp using a reverse transcription nested PCR. Briefly, RT-PCR with the first PCR round were performed using OneStep RT-PCR kit (Qiagen, Germany) and HotStar Master Mix Kit (Qiagen, Germany) respectively. For the first round, 2 µL of RNA was used in a final reaction volume of 12.5 µL using the SaV 53a+b and SaV 58 primers, and the second round of PCR was performed using the SaV 55a+b and SaV 58 primers. All primers were used at the final concentration of 500 nM (Supplementary Tables S1 and S2).

For the detection of AiV, a nested RT-PCR was performed amplifying 180 bp of 3CD-region as previously described [29]. Primers AI1 and AI2 were used with the OneStep RT-PCR kit (Qiagen, Germany) for the first PCR round and the primers AI3 and AI4 with the HotStar Master Mix Kit (Qiagen, Germany) for the second PCR round. AiV positive samples were further genotyped by using a nested RT-PCR amplifying a 520 bp fragment of the 3CD region. For the first round, 2 µL of RNA was used in a final reaction volume of 12.5 µL by using OneStep RT-PCR kit (Qiagen, Germany) with the AI68 and AI70 primers. For the second round of PCR, the HotStar Master Mix Kit (Qiagen, Germany) was used with the AI69 and AI71 primers. All primers were used at the final concentration of 200 nM (Supplementary Tables S1 and S2).

All samples were screened for AstV infections by a pan-specific AstV semi-nested RT-PCR using the primers AV89a, AV89b, AV89c, AV90a, AV90b, AV90c, and AV91, as previously described [27]. The further genotyping of AstV positive samples was done by reverse transcription nested PCR as previously described [30] using the primers AV91, AV92a, and AV93 (Supplementary Table S1).

2.4. Nucleotide Sequencing and Phylogenetic Analysis

All PCR products (NoV, SaV, AiV, and HAstV) were submitted to Sanger sequencing using the corresponding PCR primers (Supplementary Table S1). Phylogenetic analysis was performed with Geneious prime 11.0.4 and MEGA7.0.26. Norovirus sequences were submitted to the online Norovirus Tool to assign genotypes (https://www.rivm.nl/mpf/typingtool/norovirus/) (accessed on 1 December 2020). For phylogenetic analysis, nucleotide sequences were aligned with the MAFFT algorithm in Geneious prime 11.0.4. In MEGA7.0.26, trees were constructed using the best fit models of substitution pattern with the lowest BIC score (Bayesian information criterion). The reliability of the branching pattern was tested with bootstrapping (1000 replicates). The nucleotide sequences of the viral pathogens of this study are available under the following GenBank accession numbers AiV MW525344-MW525346, HAstV MW525347-MW525357, NoV-GI MW506839-MW506842

(ORF1), and MW513431-MW513437 (P2 region); NoV-GII MW513401-MW513421 (ORF1) and MW506843-MW506861 (P2 region); and SaV MW525358-MW525362.

2.5. Statistical Analysis

Statistical analysis was done by Pearson's chi-square and Fisher's exact tests using GraphPad Prism software version 6.00 for Windows. A p-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Study Population

A total of 244 stool specimens were collected from participants aged between 0 and 59 months with a median age of 14 months. Of these, 72.5% (177/244) were symptomatic children between 0 and 59 months of age with a median age of 12 months, whereas the median age of asymptomatic participants was 24 months. Among children with diarrhea, 57.1% (101/177) were male and 70.1% (124/177) lived in an urban area; compared to the healthy group in which 41.8% (28/67) were male and 61.2% (41/67) lived in an urban area (Lambaréné). Overall, 190 children of the study population were aged between 0 and 24 months, with a high proportion of symptomatic participants (81.6%; 155/190).

3.2. Detection Rate of Enteric Viruses in the Study Population

Among 177 stool samples collected from patients with diarrhea symptoms, 41 (23.2%) were positive for at least one virus, versus 10/67 (14.9%) in the healthy group (p = 0.2). NoV (14.7%; 26/177) was the most prevalent in symptomatic children, followed by AstV (7.3%; 13/177). The two remaining viruses (SaV and AiV) were detected at a lower rate of 3.4% (6/177) and 1.1% (2/177), respectively. The overall detection rate of NoV, AstV, SaV, and AiV was 9% (6/67), 4.5% (3/67), 1.5% (1/67), and 6% (4/67), respectively, among controls. Among the NoVs, NoV-GII was the most frequently detected in patients with diarrhea, whereas the detection rate was similar for NoV-GI and NoV-GII in the control group. There was no statistical significance between detection rate of enteric viruses in symptomatic and healthy children (Table 1). Of the 51 study participants who tested positive for enteric viruses, 17.6% contained more than one virus. Mixed infections were found in 6 (3.4%) children suffering from diarrhea (NoV-GI+SaV, NoV-GI+AstV, NoV-GII+AstV (n = 3) and SaV-AstV) and 3 (4.5%) cases in control group (NoV-GI+AiV, NoV GI+SaV and NoV-GII+AstV).

Table 1. Detection rate of enteric viruses among children with/without diarrhea.

Virus	Diarrhea (n = 177)	No Diarrhea (n = 67)	p-Value	Total (n = 244)
NoV	26 (14.7%)	6 (9.0%)	0.2764	32 (13.1%)
NoV GI	6 (3.4%)	3 (4.5%)	1.0000	9 (3.7%)
NoV GII	20 (11.3%)	3 (4.5%)	0.1046	23 (9.4%)
AstV	13(7.3%)	3 (4.5%)	0.5371	16 (6.6%)
SaV	6 (3.4%)	1 (1.5%)	0.6212	7 (2.9%)
AiV	2 (1.1%)	4 (6.0%)	0.1184	6 (2.5%)

Table 2 summarizes the distribution of enteric viruses in children with/without diarrhea according to age, sex, and living area. Overall, enteric viruses screened were found only in symptomatic children aged 0 to 24 months, whereas the age group 25 to 59 months was found to be more infected among controls. NoV, particularly NoV-GII, was the most frequently detected virus within the six first months of life, with 21.3% (10/47) of children with diarrhea. The age group 7 to 12 months was found to be infected by all four enteric viruses (NoV, AstV, SaV, and AiV) with detection rates of 17% (8/47), 12.8% (6/47), 2.1% (1/47), and 4.3% (2/47), respectively. No case of any enteric viruses tested in this study was found in children from 24 to 59 months presenting with diarrhea. In contrast to

the control group, symptomatic children from surrounding villages and female diarrhea cases were more infected with gastroenteritis viruses detected in this study.

Table 2. Distribution of enteric viruses in the study population according age, gender, and location.

Diarrhea Cases						
	NoV *n* (%)	**NoV GI *n* (%)**	**NoV GII *n* (%)**	**AstV *n* (%)**	**SaV *n* (%)**	**AiV *n* (%)**
Age group (Months)						
0–6 (*n* = 47)	11 (23.4)	1 (2.1)	10 (21.3)	4 (8.5)	0 (0)	0 (0)
7–12 (*n* = 47)	8 (17.0)	3 (6.4)	5 (10.6)	6 (12.8%)	1 (2.1)	2 (4.3)
13–18 (*n* = 45)	5 (11.1)	2 (4.4)	3 (6.7)	2 (4.4)	2 (4.4)	0 (0)
19–24 (*n* = 16)	2 (12.5)	0 (0)	2 (12.5)	1 (6.3)	3 (18.8)	0 (0)
25–59 (*n* = 22)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Gender						
F (*n* = 76)	13 (17.1)	2 (2.6)	11 (14.5)	9 (11.8)	4 (5.3)	1 (1.3)
M (*n* = 101)	13 (12.9)	4 (4.0)	9 (8.9)	4 (4.0)	2 (2.0)	1 (1.0)
Residence						
Surrounding villages (*n* = 53)	10 (18.9)	3 (5.7)	7 (13.2)	3 (5.7)	4 (7.5)	1 (1.9)
Lambaréné (*n* = 124)	16 (12.9)	3 (2.4)	13 (10.5)	10 (8.1)	2 (1.6)	1 (0.8)
Healthy children						
	NoV *n* (%)	**NoV GI *n* (%)**	**NoV GII *n* (%)**	**AstV *n* (%)**	**SaV *n* (%)**	**AiV *n* (%)**
Age group (Months)						
0–6 (*n* = 7)	1 (14.3)	0 (0)	1 (14.3)	0 (0)	0 (0)	0 (0)
7–12 (*n* = 8)	0 (0)	0 (0)	0 (0)	1 (12.5)	0 (0)	0 (0)
13–18 (*n* = 4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
19–24 (*n* = 16)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (6.3)
25–59 (*n* = 32)	5 (15.6)	3 (9.4)	2 (6.3)	2 (6.3)	1 (3.1)	3 (9.4)
Gender						
F (*n* = 26)	1 (3.8)	1 (3.8)	0 (0)	1 (3.8)	0 (0)	2 (7.7)
M (*n* = 41)	5 (12.2)	2 (4.9)	3 (7.3)	2 (4.9)	1 (2.4)	2 (4.9)
Residence						
Surrounding villages (*n* = 39)	3 (7.7)	0 (0)	3 (7.7)	2 (5.1)	0 (0)	3 (7.7)
Lambaréné (*n* = 28)	3 (10.7)	3 (10.7)	0 (0)	1 (3.6)	1 (3.6)	1 (3.6)

The temporal pattern of enteric viruses detected from symptomatic children during the study period is shown in Figure 1. NoV, SaV, and AstV were more frequently detected during the dry season than during the rainy season. There was, however, a significant difference in NoV occurrence between dry and rainy seasons throughout the sampling period (dry season (20.4%; 20/98) and rainy season (7.6%; 6/79) $p = 0.019$), with a notable peak of NoV infection in July 2018 and August 2019. The two cases of AiV were detected during the rainy season. The seasonal distribution of enteric viruses from asymptomatic children is not shown because samples were collected very late in the study period and did not cover at least one year of sampling.

3.3. Sequence and Phylogenetic Analyses of Gastroenteritis Viruses

3.3.1. Noroviruses

From 32 norovirus positive samples, 31 (96.9%) were genotyped. Of note, norovirus GII was most frequently detected 71% (22/31) followed by GI at 29% (9/31) (Table 3). Based on both RdRp and capsid sequences, four genotypes were identified among NoV-GII including a rare recombinant GII.P31-GII.4 New Orleans strain; for one sample, only the capsid sequence could be determined (GII.6). Overall, the common recombinant GII.P31-GII.4 Sydney strain was predominant at 45.2% (14/31). Regarding NoV-GI, four strains were genotyped on both RNA polymerase and capsid sequences, among which we found a rare recombinant (GI.P11-GI.2). In addition, four genotypes were only identified based on the capsid gene.

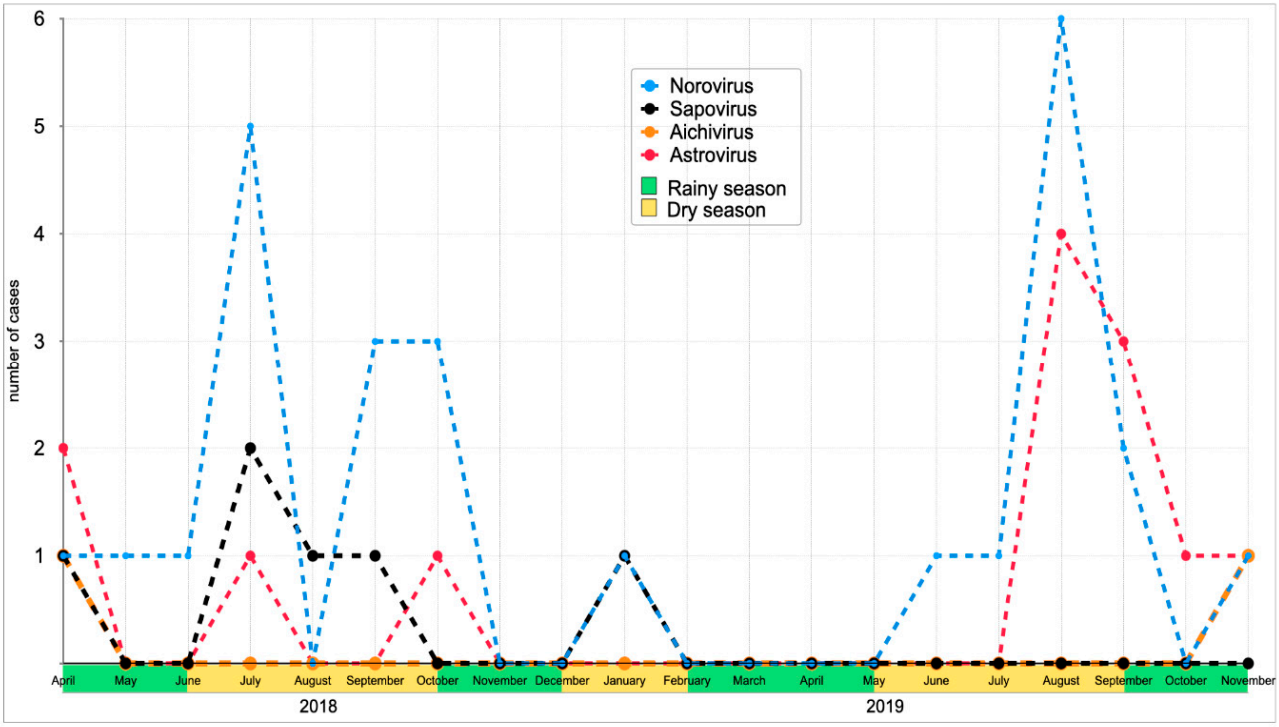

Figure 1. Seasonal distribution of enteric viruses among diarrhea cases within the sampling period, 2018–2019.

Table 3. Norovirus (NoV) polymerase and capsid genotypes obtained from the study population.

Polymerase (RdRp) Genotype	Capsid (P2 Domain) Genotype	Number (%)
Genogroup I		
GI.P3	GI.3	1 (3.2)
	GI.5	1 (3.2)
GI.P4	GI.4	1 (3.2)
GI.P5	GI.5	1 (3.2)
	GI.3	2 (6.5)
	GI.	1 (3.2)
	GI.2	1 (3.2)
GI.P11	GI.2	1 (3.2)
Genogroup II		
GII.P17	GII.17	3 (9.7)
GII.P17	GII.	1 (3.2)
GII.P31	GII.4 Syd	14 (45.2)
	GII.6	1 (3.2)
GII.P31	GII.4 NO	3 (9.7)

Phylogenetic analysis based on RNA polymerase and capsid regions for GI showed a diversity and clustering among Gabonese strains. The phylogenetic trees obtained in this study demonstrated that all NoV-GI strains genotyped either from RdRp or capsid region were grouped in different clades. Interestingly, all of these NoV-GI Gabonese strains shared a close similarity with recombinants from different geographical areas, indicating the circulation of closely related strains globally (Figure 2).

The phylogenetic analysis for NoV-GII revealed that NoV-GII Gabonese strains formed three distinct phylogenetic clusters in the two trees based on RdRp and capsid sequences, respectively. The polymerase-based tree clearly showed that 17 Gabonese strains were closely related to the reference strain GII.P31 (JX459907). The other clusters were most similar to GII.P17 (LC037415). A similarly clustering was observed with the phylogenetic tree on the capsid region. Of the four sequences which clustered in the ORF1 with the

reference strain GII.P17 (LC037415), three samples could be amplified in the P2 region. These three sequences clustered in the P2-phylogenetic tree with the reference sequence GII.17 (AY502009). Of the 17 GII.P31 sequences, 16 samples could be amplified in the P2 region. Three of these sequences clustered with the reference sequence GII.4 New Orleans (JN400623), whereas the other sequences were more related to the reference sequence GII.4 Sydney (JX459908) (Figure 3).

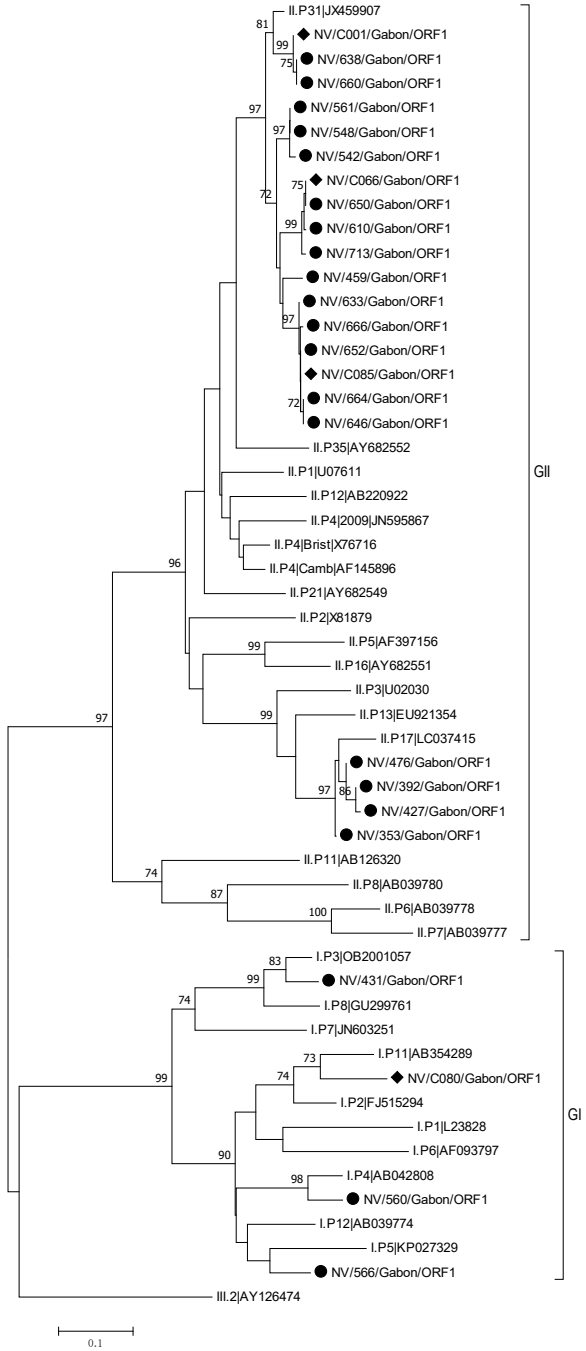

Figure 2. Phylogenetic tree of a 248 bp alignment of ORF1 region of the GI and GII NoV strains from Gabon and NoV reference sequences (accession nos. are indicated). Samples from symptomatic patients are marked with a dot and samples from asymptomatic with a rhombus. The tree was constructed using the neighbor-joining method with bootstrap test (1000 replicates) and the Kimura 2-parameter method available in MEGA7. Bootstrap values above 70 are shown. The bar indicates the nucleotide substitution per site.

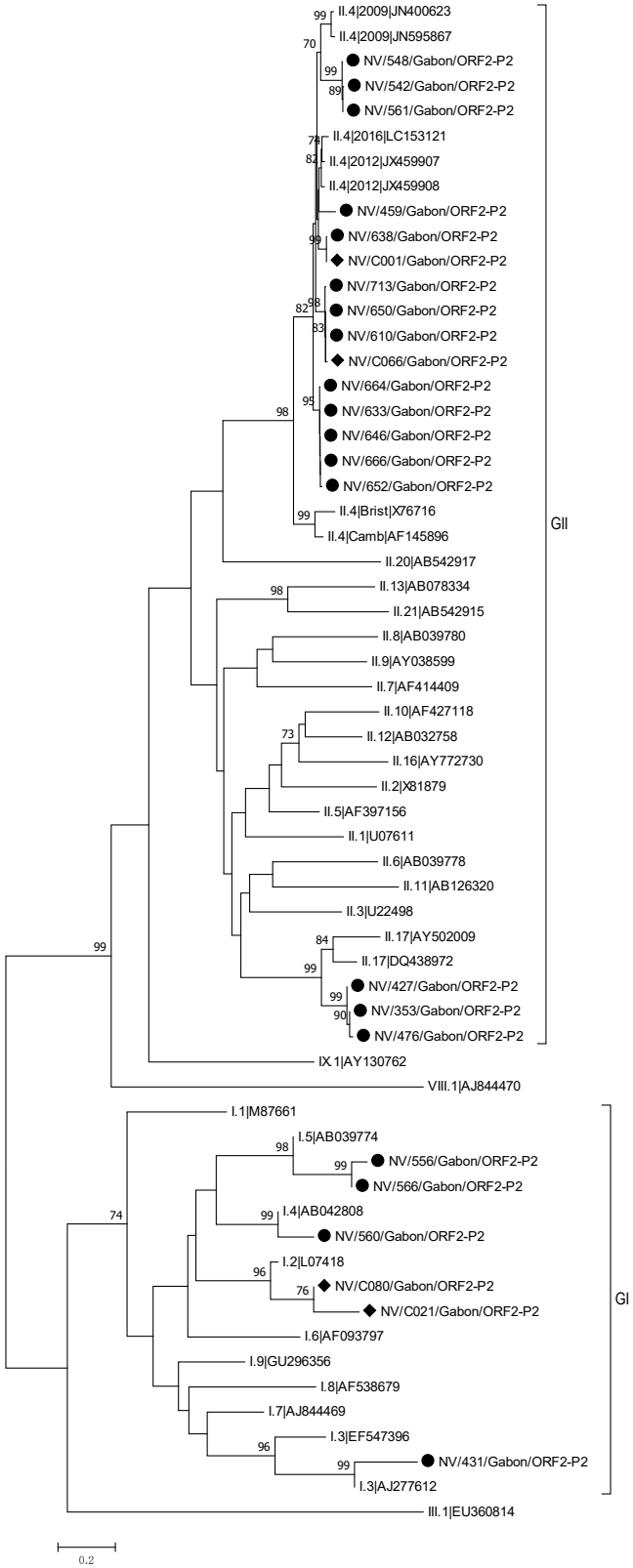

Figure 3. Phylogenetic tree of a 691 bp alignment of P2 region (ORF2) of the GI and GII NoV strains from Gabon and NoV reference sequences (accession nos. are indicated). Samples from symptomatic patients are marked with a dot and samples from asymptomatic patients with a rhombus. The tree was constructed using the neighbor-joining method with the bootstrap test (1000 replicates) and the Kimura 2-parameter method available in MEGA7. Bootstrap values above 70 are shown. The bar indicates the nucleotide substitution per site.

3.3.2. Astroviruses

This study revealed that 6.6% (16/244) of the participants had AstV infection. Sequence and phylogenetic analysis of the positive cases from a partial nucleotide sequence of ORF1b region confirmed 11 HAstV genotypes. This analysis demonstrated that samples belonged to five different HAstV genotypes. Classic HAstV were predominant with a proportion of 63.7% (7/11). Among these, three samples belonged to HAstV-5, three others belonged to HAstV-4, and the last sample belonged to HAstV-8. Within the newly HAstV, three samples belonged to HAstV-VA2 and the remaining sample belonged to MLB type 1 (MLB1) (Figure 4).

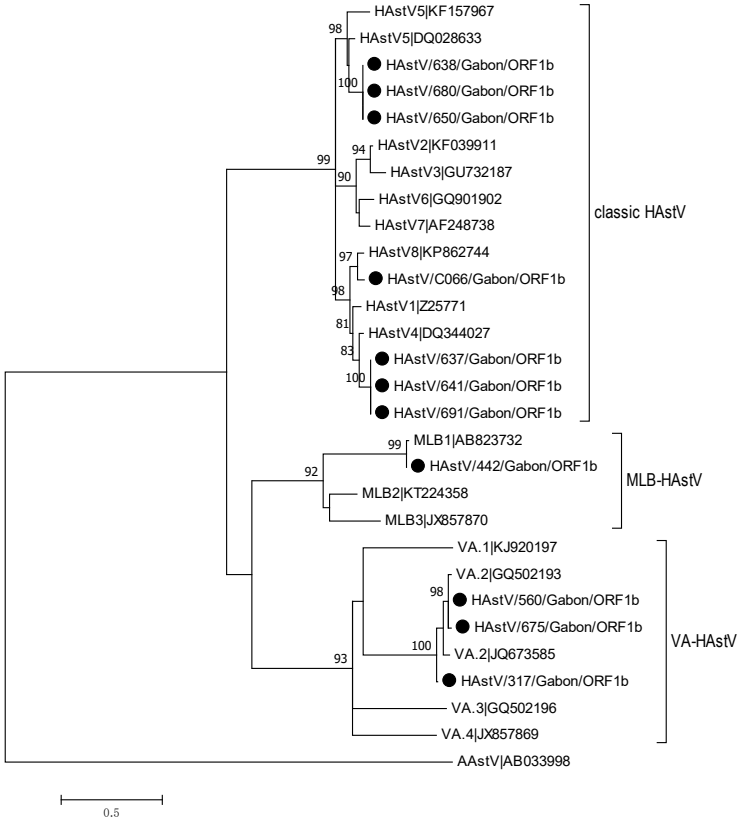

Figure 4. Phylogenetic tree of a 419 bp alignment of ORF1b region of astrovirus strains from Gabon and HAstV reference strains (accession nos. are indicated). Samples from symptomatic patients are marked with a dot and samples from asymptomatic patients with a rhombus. The tree was constructed using the maximum likelihood method with the bootstrap test (1000 replicates) and Tamura 3-parameter method available in MEGA7. The bar indicates the nucleotide substitution per site.

3.3.3. Sapoviruses

In order to characterize SaV strains, the sequence of polymerase (partial nucleotide sequence of ORF1) was phylogenetically analyzed (Figure 5). Five of all SaV positive samples were successfully genotyped and assigned into two distinct genogroups, GI and GII. Three different GI genotypes were found in our study: GI.2 (608), GI.3 (317) and GI.1 (C021 and 449). The GII strain was classified into GII.4 (524).

3.3.4. Aichiviruses

In this study, six AiV positive samples were found, confirmed by sequencing. To characterize these samples in more detail we used a prolonged PCR in the same region (3CD). Our analysis revealed that two samples belonged to genotype A and one sample belonged to genotype C, which is rare [21] (Figure 6).

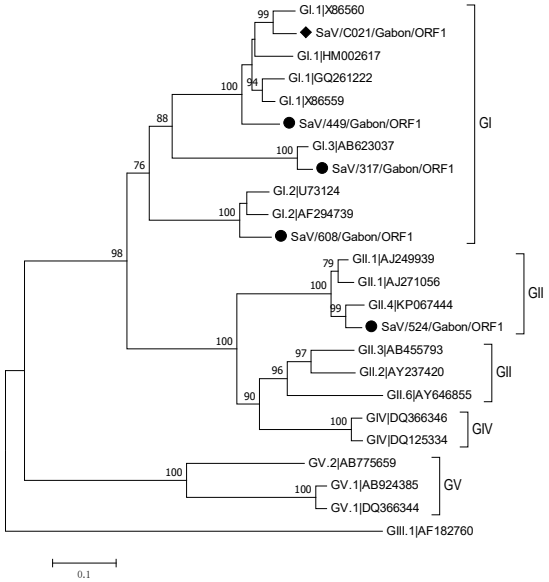

Figure 5. Phylogenetic tree of a 593 bp alignment of polymerase region (ORF1) of sapoviruses (SaV) strains from Gabon and SaV reference sequences (accession nos. are indicated). Samples from symptomatic patients are marked with a dot and samples from asymptomatic patients with a rhombus. The tree was constructed using the neighbor-joining method with the Bootstrap test (1000 replicates) and the Kimura 2-parameter method available in MEGA7. The bar indicates the nucleotide substitution per site.

Figure 6. Phylogenetic tree of a 459 bp alignment of 3CD region of aichivirus (AiV) strains from Gabon and AiV reference sequences (accession nos. are indicated). Samples from symptomatic patients are marked with a dot and samples from asymptomatic patients with a rhombus. The tree was constructed using the neighbor-joining method with the bootstrap test (1000 replicates) and Tamura 3-parameter method available in MEGA7. Bootstraps value above 70 are shown. The bar indicates the variation scale.

4. Discussion

The present study describes the detection and characterization of viral agents implicated in gastroenteritis in Gabonese children. Over the duration of the study, at least one enteric virus was detected in 20.9% of the study population, of which 23.2% (41/177) were symptomatic cases and 14.9% (10/67) in the control group. Contrary to the previous study conducted in Gabon in 2015 [25], rotaviruses and adenoviruses were not tested in this study. In accordance with previous studies, NoV was predominant, followed by HAstV, SaV, and AiV [25,31]. AiV, reported for the first time in Gabon and in Central Africa, was found with a lower detection rate in symptomatic cases (1.1%) than in healthy children (6%). This finding is in line with previous reports, suggesting that AiV may not an important viral gastroenteritis agent [32]. In our study, more than one virus was detected in 17.6% of studied children, while higher detection rates of 35.5% were found in Cameroon, in Gabon (33.7%), and mixed infections rates of 6.7% and 2.8% were respectively reported in Nigeria and Canada [25,33–35]. In this regard, we clearly noticed a disparity of mixed infections rate amongst studies, which could be explained by the number of targeted virus species, sample size, sampling (repeat sampling), storage, and the detection methods [34].

Our findings demonstrated that children were carried enteric viruses implicated in diarrhea in the 0 to 24 months age group. This is consistent with previous reports emphasizing that maternal antibodies transmitted during breastfeeding are not sufficient to protect against gastroenteritis viruses during the first 24 months of life [36,37]. Furthermore, our results suggest that the presence of viruses in this age group could be due to direct contact with people who experienced gastroenteritis symptoms, contaminated water, or food [38]. This could be explained by the fact that at this age, children crawl, are carried by people, and are likely to touch any contaminated object.

A significant seasonal pattern of NoV was observed in this study with major peaks during dry seasons. This finding is similar to that reported in Cameroon showing a peak of NoV in the beginning of wet season [33]. HAstV was detected with a major peak during the dry season, which is similar to a report from Nigeria [34]. Regarding SaV and AiV, we observed a relative seasonal distribution throughout the sampling period with a peak during the dry season for SaV and the occurrence of AiV in April and November. These findings are in line with the results described in previous studies reporting SaV seasonal distribution with a peak in the dry season, and high seasonal distribution of AiV infections in January, February, and December [22,39].

In this study, NoV was the most common virus detected in symptomatic children (14.7%) and the control group (9%), with a high rate of NoV-GII among participants with diarrhea (11.3%). This predominance was also reported in other studies showing that NoV was the second most prevalent virus after rotavirus compared to other enteric viruses. In addition, our results are comparable to findings in Cameroonian and Burkinabe studies reporting NoV cases in healthy children, albeit with a considerably lower detection rate in our study [33,40]. More generally, we found a lower prevalence of NoV in our study compared to that reported in all previous studies from neighboring countries [33,37]. As observed elsewhere, GI strains were less frequently detected and had a higher diversity than GII genogroups [26,30,41]. The predominance of the GII.4 variants in this study may reinforce the hypothesis and emphasize the need to explore whether new GII.4 variants possibly arise in Africa where some of the GII.4 variants circulated earlier [24]. Moreover, the fact that NoV GII.4 was found to be predominant in our study, whereas in various African countries the GII.17 strain emerged and was mostly detected in Kenya, underlines that dynamic evolution of NoVs might occur unexpectedly [42,43]. The most prevalent NoV genotype was GII.P31-GII.4 Sydney, with a 45.2% detection rate. This finding is consistent with the results reported in Germany, Brazil, and China [26,44,45]. Surprisingly, we did not find that the emerging recombinant strain GII.P16-GII.4 replaced GII.P31-GII.4 Sydney around the world [46,47].

Our results confirmed that HAstV (7.3%) is less common in children with acute gastroenteritis compared to NoV. This finding is relatively similar to the detection rate of

HAstV found in children with diarrhea in Nigeria (6.8%) [30] and Burkina Faso (4.9%) [40]. HAstV showed a high diversity in this study, clustering with newly identified HAstV (HAstV-VA2 and MLB1) and classic HAstV (HAstV5, HAstV4, and HAstV8). Both HAstV genotypes (HAstV-VA2 and MLB1) were for the first time reported in Gabon. Interestingly, we did not find the most common HAstV-1 in this study but only uncommon HAstV-5 and rare HAstV-8 genotypes were identified.

SaV was found at a low detection rate of 3.4% in children with diarrhea. This is in line with a previous report revealing a range of 3% to 17% of SaV infections among children with gastroenteritis in high- and low-income countries [48].This result also shows that SaV is less detected in gastroenteritis cases than NoV. The circulation of SaV genogroups shows variability with SaV-GI. Moreover, SaV-GI.1 was detected in two individuals whereas only one case of each other GI genotypes was detected among the study population. In contrast, SaV-GII was less frequent in this present study than in Burkinabe children with diarrhea where SaV-GII appeared to be predominant [40].

A low prevalence of AiV A strains was found in this study with more cases in healthy children as reported elsewhere [32]. This finding is concordant with several studies that reported low prevalence of this enteric virus [49,50]. All detected AiV strains belonged to genotype A and C. In contrast to previous findings reporting the presence of AiV A in children with gastroenteritis [49,51], the AiVs A detected in our study were from healthy children. This reinforces the need of further studies to elucidate the full spectrum of clinical symptoms of AiV. The rare AiV C reported only in Burkina Faso [40] and France [52] from children with gastroenteritis was also identified in a symptomatic child in our study.

Overall, due to the close relationship between the Gabonese enteric viruses and the reference strains, our results revealed that the same strains are circulating around the world. This could be explained by the migration of foreigners that facilitate the importation of infectious agents [53]. In addition, the relative proportions of viruses reported here suggests a possible problem of life conditions, such as water or food contamination, or the sociodemographic level of population. It is therefore important to understand the local epidemiology of these viruses, which will provide information on the disease transmission and contribute to the development of vaccines and treatment strategies.

A limitation of our study is the small size of control group. Furthermore, the fact that the rotavirus and adenovirus were not screened in this study underestimated the information about virus co-infection. In addition, seasonal distribution of enteric viruses among asymptomatic children was not shown because of the sampling that started towards the second year.

5. Conclusions

In conclusion, this study shows an important rate of viral detection (23.2%) among Gabonese children <5 years with diarrhea. NoV was the predominant virus associated with diarrhea. To the best of our knowledge, this study provides the first report on the detection of AiV in Central Africa, particularly in Gabon.

Molecular characterization reported a great diversity of enteric virus strains. Thus, our data would be useful for better management of preventive strategies such as vaccination.

Supplementary Materials: The following are available online at https://www.mdpi.com/1999-4915/13/4/545/s1, Table S1: primer and probes for detection and genotyping of different viral GE pathogens used in the study and Table S2: Hitherto unpublished PCR programs and primers used in this study.

Author Contributions: Conceptualization, S.N. and G.P.M.; methodology, S.N.; software, S.N.; validation, S.N., G.P.M. and C.-T.B.; Specimen collection, G.P.M. and P.A.N.-M.; data curation, S.N. and G.P.M.; writing—original draft preparation, G.P.M. and S.N.; writing—review and editing, P.A.N.-M., M.M.N., C.-T.B., P.G.K., S.B., D.E., B.M., T.P.V., A.A.A., S.N. and G.P.M.; funding acquisition, A.A.A., P.G.K., and T.P.V. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study protocol was approved (by the Institutional Ethical Committee of the Centre of Medical Research in Lambaréné (CERMEL) (CEI-CERMEL: 003/2017). Ethics approval was obtained on 1 March 2018.

Informed Consent Statement: Written informed consent was obtained from parents or legal representatives of the participants.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Gautam, R.; Lyde, F.; Esona, M.D.; Quaye, O.; Michael, D.; Viruses, R. Detection of Rotavirus Antigen in Stool Specimens. *J. Clin. Virol.* **2013**, *58*, 292–294. [CrossRef]
2. Black, R.E.; Cousens, S.; Johnson, H.L.; Lawn, J.E.; Rudan, I.; Bassani, D.G.; Jha, P.; Campbell, H.; Walker, C.F.; Cibulskis, R.; et al. Global, regional, and national causes of child mortality in 2008: A systematic analysis. *Lancet* **2010**, *375*, 1969–1987. [CrossRef]
3. Li, V. The Epidemiology of Noroviruses in Ghana: A Case Study of Norovirus Detection. *J. Glob. Health* **2013**, *3*, 11–14.
4. Corcoran, M.S.; Van Well, G.T.J.; Van Loo, I.H.M. Diagnosis of viral gastroenteritis in children: Interpretation of real-time PCR results and relation to clinical symptoms. *Eur. J. Clin. Microbiol. Infect. Dis.* **2014**, *33*, 1663–1673. [CrossRef]
5. Sdiri-Loulizi, K.; Gharbi-Khélifi, H.; De Rougemont, A.; Chouchane, S.; Sakly, N.; Ambert-Balay, K.; Hassine, M.; Guédiche, M.N.; Aouni, M.; Pothier, P. Acute infantile gastroenteritis associated with human enteric viruses in Tunisia. *J. Clin. Microbiol.* **2008**, *46*, 1349–1355. [CrossRef]
6. Ouyang, Y.; Ma, H.; Jin, M.; Wang, X.; Wang, J.; Xu, L.; Lin, S.; Shen, Z.; Chen, Z.; Qiu, Z.; et al. Etiology and epidemiology of viral diarrhea in children under the age of five hospitalized in Tianjin, China. *Arch. Virol.* **2012**, *157*, 881–887. [CrossRef] [PubMed]
7. Parashar, U.D.; Hummelman, E.G.; Bresee, J.S.; Miller, M.A.; Glass, R.I. Global illness and deaths caused by rotavirus disease in children. *Emerg. Infect. Dis.* **2003**, *9*, 565–572. [CrossRef] [PubMed]
8. Tate, J.E.; Burton, A.H.; Boschi-Pinto, C.; Parashar, U.D. Global, Regional, and National Estimates of Rotavirus Mortality in Children <5 Years of Age, 2000–2013. *Clin. Infect. Dis.* **2016**, *62* (Suppl. 2), S96–S105. [CrossRef]
9. Arnold, M.M. Rotavirus Vaccines: Why Continued Investment in Research Is Necessary. *Curr. Clin. Microbiol. Rep.* **2018**, *5*, 73–81. [CrossRef]
10. da Silva Poló, T.; Peiró, J.R.; Mendes, L.C.N.; Ludwig, L.F.; De Oliveira-Filho, E.F.; Bucardo, F.; Huynen, P.; Melin, P.; Thiry, E.; Mauroy, A. Human norovirus infection in Latin America. *J. Clin. Virol.* **2016**, *78*, 111–119. [CrossRef]
11. Bartsch, S.M.; Lopman, B.A.; Ozawa, S.; Hall, A.J.; Lee, B.Y. Global economic burden of norovirus gastroenteritis. *PLoS ONE* **2016**, *11*, e0151219. [CrossRef]
12. Carmona-Vicente, N.; Vila-Vicent, S.; Allen, D.; Gozalbo-Rovira, R.; Iturriza-Gómara, M.; Buesa, J.; Rodríguez-Díaz, J. Characterization of a Novel Conformational GII.4 Norovirus Epitope: Implications for Norovirus-Host Interactions. *J. Virol.* **2016**, *90*, 7703–7714.
13. Robilotti, E.; Deresinski, S.; Pinsky, B.A. Norovirus. *Clin. Microbiol. Rev.* **2015**, *28*, 134–164. [CrossRef] [PubMed]
14. Chhabra, P.; de Graaf, M.; Parra, G.I.; Chan, M.C.-W.; Green, K.; Martella, V.; Wang, Q.; White, P.A.; Katayama, K.; Vennema, H.; et al. Erratum: Updated classification of norovirus genogroups and genotypes (Journal of General Virology (2020) 100 (1393–1406) doi:10.1099/jgv.0.001318). *J. Gen. Virol.* **2020**, *101*, 893. [CrossRef]
15. Tohma, K.; Lepore, C.J.; Gao, Y.; Ford-Siltz, L.A.; Parra, G.I. Population Genomics of GII.4 Noroviruses Reveal Complex Diversification and New Antigenic Sites Involved in the Emergence of Pandemic Strains. *mBio* **2019**, *10*, e02202-19. [CrossRef] [PubMed]
16. Johnson, C.; Hargest, V.; Cortez, V.; Meliopoulos, V.A.; Schultz-Cherry, S. Astrovirus pathogenesis. *Viruses* **2017**, *9*, 22. [CrossRef]
17. Bosch, A.; Pintó, R.M.; Guix, S. Human Astroviruses. *Clin. Microbiol. Rev.* **2014**, *27*, 1048–1074. [CrossRef]

18. Vu, D.-L.; Bosch, A.; Pintó, R.M.; Guix, S. Epidemiology of classic and novel human astrovirus: Gastroenteritis and beyond. *Viruses* **2017**, *9*, 33. [CrossRef]
19. Magwalivha, M.; Kabue, J.-P.; Traore, A.N.; Potgieter, N. Prevalence of Human Sapovirus in Low and Middle Income Countries. *Adv. Virol.* **2018**, *2018*, 1–12. [CrossRef] [PubMed]
20. Oka, T.; Wang, Q.; Katayama, K.; Saif, L.J. Comprehensive review of human sapoviruses. *Clin. Microbiol. Rev.* **2015**, *28*, 32–53. [CrossRef]
21. Ambert-Balay, K.; Lorrot, M.; Bon, F.; Giraudon, H.; Kaplon, J.; Wolfer, M.; Lebon, P.; Gendrel, D.; Pothier, P. Prevalence and genetic diversity of Aichi virus strains in stool samples from community and hospitalized patients. *J. Clin. Microbiol.* **2008**, *46*, 1252–1258. [CrossRef] [PubMed]
22. Taghinejad, M.; Ghaderi, M.; Mousavi-Nasab, S.D. Aichivirus with acute gastroenteritis in Iran. *Pediatric Infect. Dis. J.* **2020**, *39*, 576–579. [CrossRef] [PubMed]
23. Kotloff, K.L. The Burden and Etiology of Diarrheal Illness in Developing Countries. *Pediatric Clin. N. Am.* **2017**, *64*, 799–814. [CrossRef]
24. Mans, J.; Armah, G.E.; Steele, A.D.; Taylor, M.B. Norovirus epidemiology in Africa: A review. *PLoS ONE* **2016**, *11*, e0146280. [CrossRef]
25. Lekana-Douki, S.E.; Kombila-Koumavor, C.; Nkoghe, D.; Drosten, C.; Drexler, J.F.; Leroy, E.M. Molecular epidemiology of enteric viruses and genotyping of rotavirus A, adenovirus and astrovirus among children under 5 years old in Gabon. *Int. J. Infect. Dis.* **2015**, *34*, 90–95. [CrossRef]
26. Niendorf, S.; Faber, M.; Tröger, A.; Hackler, J.; Jacobsen, S. Diversity of noroviruses throughout outbreaks in Germany 2018. *Viruses* **2020**, *12*, 1157. [CrossRef] [PubMed]
27. Jacobsen, S.; Höhne, M.; Marques, A.M.; Beslmüller, K.; Bock, C.-T.; Niendorf, S. Co-circulation of classic and novel astrovirus strains in patients with acute gastroenteritis in Germany. *J. Infect.* **2018**, *76*, 457–464. [CrossRef] [PubMed]
28. Höhne, M.; Niendorf, S.; Marques, A.M.; Bock, C.-T. Use of sequence analysis of the P2 domain for characterization of norovirus strains causing a large multistate outbreak of norovirus gastroenteritis in Germany 2012. *Int. J. Med. Microbiol.* **2015**, *305*, 612–618. [CrossRef]
29. Oh, D.-Y.; Silva, P.A.; Hauroeder, B.; Diedrich, S.; Cardoso, D.D.P.; Schreier, E. Molecular characterization of the first Aichi viruses isolated in Europe and in South America. *Arch. Virol.* **2006**, *151*, 1199–1206. [CrossRef]
30. Japhet, M.O.; Famurewa, O.; Adesina, O.; Opaleye, O.; Wang, B.; Höhne, M.; Bock, C.; Marques, A.M.; Niendorf, S. Viral gastroenteritis among children of 0–5 years in Nigeria: Characterization of the first Nigerian aichivirus, recombinant noroviruses and detection of a zoonotic astrovirus. *J. Clin. Virol.* **2019**, *111*, 4–11. [CrossRef]
31. Shen, X.-X.; Qiu, F.-Z.; Li, G.-X.; Zhao, M.-C.; Wang, J.; Chen, C.; Zhao, L.; Qi, J.-J.; Liu, H.; Zhang, Y.; et al. A case control study on the prevalence of enterovirus in children samples and its association with diarrhea. *Arch. Virol.* **2018**, *164*, 63–68. [CrossRef] [PubMed]
32. Han, T.-H.; Park, S.H.; Hwang, E.-S.; Reuter, G.; Chung, J.-Y. Detection of Aichi virus in South Korea. *Arch. Virol.* **2014**, *159*, 1835–1839. [CrossRef] [PubMed]
33. Ayukekbong, J.; Lindh, M.; Nenonen, N.; Tah, F.; Nkuo-Akenji, T.; Bergström, T. Enteric viruses in healthy children in cameroon: Viral load and genotyping of norovirus strains. *J. Med. Virol.* **2011**, *83*, 2135–2142. [CrossRef] [PubMed]
34. Arowolo, K.O.; Ayolabi, C.I.; Lapinski, B.; Santos, J.S.; Raboni, S.M. Epidemiology of enteric viruses in children with gastroenteritis in Ogun State, Nigeria. *J. Med. Virol.* **2019**, *91*, 1022–1029. [CrossRef] [PubMed]
35. Leblanc, D.; Inglis, G.D.; Boras, V.F.; Brassard, J.; Houde, A. The prevalence of enteric RNA viruses in stools from diarrheic and non-diarrheic people in southwestern Alberta, Canada. *Arch. Virol.* **2017**, *162*, 117–128. [CrossRef]
36. Cannon, J.L.; Lopman, B.A.; Payne, D.C.; Vinjé, J. Birth Cohort Studies Assessing Norovirus Infection and Immunity in Young Children: A Review. *Clin. Infect. Dis.* **2019**, *69*, 357–365. [CrossRef]
37. Louya, V.M.; Vouvoungui, C.; Koukouikila-Koussounda, F.; Veas, F.; Kobawila, S.C.; Ntoumi, F.; Mikounou, V.; Charles, K.S. Molecular characterization of norovirus infection responsible for acute diarrhea in Congolese hospitalized children under five years old in Brazzaville, Republic of Congo. *Int. J. Infect. Dis.* **2019**, *88*, 41–48. [CrossRef]
38. Wu, C.Y.; Chi, H.; Liu, C.-C.; Huang, Y.-C.; Huang, Y.-C.; Lin, H.-C.; Ho, Y.-H.; Huang, L.-M.; Huang, C.-Y.; Shih, S.-M.; et al. Clinical characteristics and risk factors for children with norovirus gastroenteritis in Taiwan. *J. Microbiol. Immunol. Infect.* **2020**. [CrossRef]
39. Ouedraogo, N.; Ngangas, S.M.T.; Bonkoungou, I.J.O.; Tiendrebeogo, A.B.; Traore, K.A.; Sanou, I.; Traore, A.S.; Barro, N. Temporal distribution of gastroenteritis viruses in Ouagadougou, Burkina Faso: Seasonality of rotavirus. *BMC Public Health* **2017**, *17*, 1–8. [CrossRef]
40. Ouédraogo, N.; Kaplon, J.; Bonkoungou, I.J.O.; Traoré, A.S.; Pothier, P.; Barro, N.; Balay, K.A. Prevalence and genetic diversity of enteric viruses in children with diarrhea in Ouagadougou, Burkina Faso. *PLoS ONE* **2016**, *11*, e0153652. [CrossRef]
41. Bucardo, F.; Reyes, Y.; Svensson, L.; Nordgren, J. Predominance of norovirus and sapovirus in nicaragua after implementation of universal rotavirus vaccination. *PLoS ONE* **2014**, *9*, e98201. [CrossRef]
42. El Qazoui, M.; Oumzil, H.; Baassi, L.; El Omari, N.; Sadki, K.; Amzazi, S.; Benhafid, M.; El Aouad, R. Rotavirus and Norovirus infections among acute gastroenteritis children in Morocco. *BMC Infect. Dis.* **2014**, *14*, 300. [CrossRef] [PubMed]

43. Kiulia, N.M.; Mans, J.; Mwenda, J.M.; Taylor, M.B. Norovirus GII.17 Predominates in Selected Surface Water Sources in Kenya. *Food Environ. Virol.* **2014**, *6*, 221–231. [CrossRef] [PubMed]
44. Kuang, X.; Teng, Z.; Zhang, X. Genotypic prevalence of norovirus GII in gastroenteritis outpatients in Shanghai from 2016 to 2018. *Gut Pathog.* **2019**, *11*, 1–11. [CrossRef] [PubMed]
45. Guarines, K.M.; Mendes, R.P.G.; de Magalhães, J.J.F.; Pena, L. Norovirus-associated gastroenteritis, Pernambuco, Northeast Brazil, 2014–2017. *J. Med. Virol.* **2020**, *92*, 1093–1101. [CrossRef]
46. Hasing, M.E.; Lee, B.E.; Qiu, Y.; Xia, M.; Pabbaraju, K.; Wong, A.; Tipples, G.; Jiang, X.; Pang, X.L. Changes in norovirus genotype diversity in gastroenteritis outbreaks in Alberta, Canada: 2012–2018. *BMC Infect. Dis.* **2019**, *19*, 1–9. [CrossRef] [PubMed]
47. Hernandez, J.M.; Silva, L.D.; Junior, E.C.S.; Cardoso, J.F.; Reymão, T.K.A.; Portela, A.C.R.; De Lima, C.P.S.; Teixeira, D.M.; Lucena, M.S.S.; Nunes, M.R.T.; et al. Evolutionary and Molecular Analysis of Complete Genome Sequences of Norovirus From Brazil: Emerging Recombinant Strain GII.P16/GII.4. *Front. Microbiol.* **2020**, *11*, 1–10. [CrossRef] [PubMed]
48. Becker-Dreps, S.; Bucardo, F.; Vinjé, J. Sapovirus: An important cause of acute gastroenteritis in children. *Lancet Child Adolesc. Health* **2019**, *3*, 758–759. [CrossRef]
49. Kaikkonen, S.; Räsänen, S.; Rämet, M.; Vesikari, T. Aichi virus infection in children with acute gastroenteritis in Finland. *Epidemiol. Infect.* **2010**, *138*, 1166–1171. [CrossRef]
50. Nielsen, A.C.Y.; Gyhrs, M.L.; Nielsen, L.P.; Pedersen, C.; Böttiger, B. Gastroenteritis and the novel picornaviruses aichi virus, cosavirus, saffold virus, and salivirus in young children. *J. Clin. Virol.* **2013**, *57*, 239–242. [CrossRef]
51. Jonsson, N.; Wahlström, K.; Svensson, L.; Serrander, L.; Lindberg, A.M. Aichi virus infection in elderly people in Sweden. *Arch. Virol.* **2012**, *157*, 1365–1369. [CrossRef] [PubMed]
52. Rivadulla, E.; Romalde, J.L. Correction to: A Comprehensive Review on Human Aichi Virus. *Virol. Sin.* **2020**, *12250*, 15782.
53. Widdowson, M.-A.; Sulka, A.; Bulens, S.N.; Beard, R.S.; Chaves, S.S.; Hammond, R.; Salehi, E.D.; Swanson, E.; Totaro, J.; Woron, R.; et al. Norovirus and foodborne disease, United States, 1991–2000. *Emerg. Infect. Dis.* **2005**, *11*, 95–102. [CrossRef] [PubMed]

3. DISCUSSION

3.1. Pathogens found in stool of children under five presenting with diarrhea

Since 2017, the WHO response to diarrheal diseases is to promote national policies and actions that improve case management of diarrhea. Therefore, to gather initial information about diarrheal infections, in Chapter 1, we screened eight pathogens, including viruses (adenovirus and rotavirus), parasites (*Cryptosporidium* spp., *Cyclospora cayetanensis* and *Giardia lamblia*) and bacteria (*Salmonella* spp., ETEC and EIEC/*Shigella)* in young children presenting as outpatients with diarrhea. Our data reports the presence of at least one of the tested infectious agents in 50 % of the analysed samples. EIEC/*Shigella* and ETEC were the most frequently detected agents, followed by *G. lamblia*, *Cryptosporidium* spp. and rotavirus. Aside from the different diagnostic methods used, our results are similar to the findings of the Global Enteric Multicenter Study (GEMS) aimed to identify the aetiology and population-based burden of paediatric diarrheal disease in sub-Saharan Africa and south Asia, in which *Shigella* was identified as the number one pathogen and ETEC ranked fourth. Rota- and adenovirus were the second and third most prevalent enteric pathogens in the GEMS study [102].

EIEC/Shigella with a prevalence of 30 %, was twofold more frequent than reported by Sebastien Breurec *et al.,* in 2016 in the Central African Republic [103]. Compared to a study conducted in 2003 in the same setting [104], in which no case of EIEC/*Shigella* was found, our finding could indicate a new emergence of *E. coli* (EIEC/*Shigella*) pathotype in the study population after twenty years. Furthermore, this pathotype showed a significant association with the age group7-59 months.

ETEC pathotypes characterized by the expression of either the *elt* or *est* gene or both were detected in 25 % of the study population. This proportion is higher compared to what was found in Karnataka (India) (13.6 %), a previous survey in Lambaréné (4.67 %) and Port Blair (Andaman & Nicobar Islands) (10.3 %) [104–106]. The emergent EIEC/*Shigella* and ETEC pathotypes were frequently found

in combination. The occurrence of coinfections could be explained by the plasticity of the *E. coli* genome, which has the potential of undergoing continuous rearrangements. Moreover, horizontal gene transfer by mobile elements plays a major role in genome flexibility [105,107].

Cryptosporidium spp. was identified in 13 % of children presenting with diarrhea. This finding confirms the recent discovery of *Cryptosporidium spp.* as an important pathogen in infantile diarrhea. Our results showed that the prevalence of *Cryptosporidium spp.* varies considerably between studies. For instance, a study in Angola found *Cryptosporidium* spp. in 30 % of children with diarrhea [108], while in Kenya and Mozambique lower proportions were reported (4% and 0.6 % respectively) [109,110]. Another study from Libreville (Gabon) found a prevalence of 24 % among children aged 0-2 years with diarrhea [111].

Rotavirus was found in 10% of our patients, which is a lower proportion than what was previously reported in other parts of Gabon (27 %) [112], in Angola (25 %) [108] and in the Central African Republic (CAR) (40 %) [103]. Each of these studies was conducted in the same age group with similar inclusion criteria. This variability of proportions across the studies could be explained by many factors (locations, sample size) although in our case the main reason could be the use of a low-sensitivity method (RDT) for the detection of rotavirus, while most of the studies used PCR-based methods, which offer a high sensitivity [113]. Moreover, our results showed a significant negative association between the presence of rotavirus and the age group 7-59 months.

Although there is still doubt on the role of *Giardia* as an etiological agent responsible for diarrhea [114], in our study we found *G. lamblia* in 13 % of children with a significant association in the first twelve months of life. This number is important when considering a report from another Central African country (Central African Republic) where low numbers of *Giardia* were identified among symptomatic children [103]. Our finding reinforces the need to clearly determine whether *Giardia* is a cause of diarrhea or may just be an accompanying colonisation as suggested elsewhere [1,103].

Adenovirus (3.3 %); *C. cayetanensis* (3.3 %) and *Salmonella spp.* (2.1 %), were the least frequently detected pathogens among the study population. Other studies revealed a high prevalence of adenovirus, even ranking it as the third most common pathogen found in diarrheal stool [115], a finding which could not be reproduced in this study. The number of *Salmonella spp.* was lower than what was previously reported in Libreville (20.2 %; 98/485) and in Asante Akim North municipality (Ghana) (6 %) [116,117]. Our results could be explained by the fact that we used the poorly sensitive method (standard culture). In addition, this low prevalence of *Salmonella* in our study area might also be attributed to several factors impacting non typhoidal salmonella levels in food and water, which play a major role in human exposure to infection. These factors include climate, food animal production practices, the level of spread of specific serotypes in environmental reservoirs and the availability of food animal vaccination programs [118].

Overall, 33 % of symptomatic children were found to be infected with more than one pathogen. EIEC/*Shigella*, ETEC and rotavirus were the most frequently identified in combination. These co-infections in diarrheal disease could be explained by the fact that the majority of gastrointestinal organisms share the same transmission route (faecal-oral route) [117]. Moreover, the high number of co-infections observed in our data are in line with findings of other studies [1,119], The effects of co-infections on gastroenteritis cases are still discussed controversially. Several reports highlighted that no specific clinical sign of gastroenteritis is significantly related to the presence of co-infections [120,121], while others have previously linked mixed infections to the severity of symptoms [122]. In this regard, there is a great need for systematic exploration of the effects of co-infection on symptom severity and duration.

The use of non-molecular diagnostic tools, which have a low sensitivity for pathogen detection, and the absence of a control group are the main limitations of this study. This precludes any firm attribution of the identified pathogens to diarrheal disease. Theoretically, to estimate the burden of diarrhea related to a specific pathogen by calculating attributable fractions, one needs to know the prevalence of the pathogen in a population of patients suffering from diarrheal

diseases and the prevalence in a control group. In addition, the association between the quantity of pathogens and disease severity can help to attribute a symptom such as diarrhea to a pathogen.

This chapter raises new questions on the effect of infections with multiple pathogens on disease expression and the cause of the emergence of EIEC/*Shigella* in the study site. These findings serve as baseline for public health stakeholders and will guide future studies.

3.2. Evaluation of Cer Test Crypto immunochromatographic test for the detection of Cryptosporidium species in four sub-Saharan African countries

Since *Cryptosporidium* was included on the World Health Organization list of Neglected Diseases Initiative, international and national investments in prevention and control have increased. This includes a test strategy requiring a rapid, low technology, reliable, and affordable diagnostic test that can be used near the point of care, and that is suitable for use. Therefore, several rapid diagnostic tests have been developed for the detection of *Cryptosporidium* and subsequently assessed in diagnostic studies. The reported sensitivity and specificity were inconsistent between immunochromatographic test (ICT) brands (47.2 %, 62.4 %, 68.8 % and 70.6 % for Crypto-Strip, RIDA Quick, Remel-Xpect, and ImmunoCard STAT!) [123], although ICTs for cryptosporidiosis could be a potentially useful test because of its specificity.

In this regard, the second chapter of my dissertation documents that the newly commercialized Cer Test Crypto has insufficient diagnostic performance for the detection of *Cryptosporidium spp.* in four sub-Saharan African countries. Our findings are not in accordance with a previous study evaluating different RDT brands for the detection of *Cryptosporidium* [124,125]. The low sensitivity of Cer Test Crypto immunochromatographic might be attributed to low density *Cryptosporidium* infections in our population. Thus, a large proportion of low-density infection would lead to an important underestimate of the disease burden, while accurate detection of *Cryptosporidium* is important to obtain incidence and transmission dynamics in distinct areas as well as to identify sources of

transmission. Furthermore, our study demonstrated an overall low positive predictive value of 61.3 %, that could be due to the low prevalence of *Cryptosporidium* infection. The proportion of false positivity might also explain the low positive predictive value as shown by a study evaluating a detection tool for *Plasmodium falciparum* [126]. Regarding the differing percentages between tests, our study is in line with previous studies that showed the inaccuracy of immunoassays versus PCR methods [127–129].

In our investigation, a composite of PCRs was considered as a reference method. In fact, we identified four different *Cryptosporidium* genotypes (*C. hominis*, *C. parvum*, *C. meleagridis* and *C. xiaoi/bovis*). The evaluation of Cer Test Crypto RDT versus a composite reference standard of qPCR and RFLP-PCR, showed minimal changes in the RDT sensitivity when comparing different sensitivities in each study site for the detection of *Cryptosporidium spp.* and the detection of the two most common *Cryptosporidium spp.* in humans (*C. parvum* and *C. hominis*). This does not support the hypothesis that the low sensitivity of RDTs for cryptosporidiosis may also be partly due to the fact that the antibodies used to detect the parasites are species-specific and not genus-specific.[125]. Furthermore, the low performance of this RDT could be explained by the occurrence of antigen subtypes and lack of surface antigens [130]. Nevertheless, this RDT with a high specificity is reliable for detecting *Cryptosporidium*-free samples with 93 % of confidence.

This chapter provides key insights into the evolutionary development of diagnostic tools for the detection of *Cryptosporidium*. Therefore, it is desirable that an improved Cer Test Crypto RDT and ICTs in general will be developed in the near future avoiding the drawbacks of the currently available RDTs.

3.3. Rotavirus strain diversity and VP7/VP4 antigenic epitopes comparison between Gabonese and vaccine rotaviruses

Rotavirus vaccination is recommended by WHO as part of a comprehensive strategy to control diarrheal illnesses (WHO, 2013). However, in Gabon no

national immunization program for RVA has yet been implemented. This chapter attempted to investigate the diversity of rotavirus strains and provide information on the genetic and antigenic variation in the VP4 and VP7 protein of circulating strains that may affect the effectiveness of rotavirus vaccines upon their introduction in the country.

Our study reported 55 % (98/177) of rotavirus-related hospital admissions and 21 % (14/67) of rotavirus infections detected in asymptomatic children. This significant rate of RVA positive cases associated with diarrhea is higher than what was previously reported by Lekana et *al.* in 2015 (27.1 %) [112]. This difference in the proportion of rotavirus revealed a different distribution in some parts of the country. As almost all the studies indicate, rotavirus was more prevalent among participants with diarrhea than among healthy participants, although the rate was slightly higher than in previous studies. This is consistent with two previous studies, including one in Nigeria, which reported a rate of 55.9 % rotavirus positive in subjects with diarrhea, while no rotavirus was found in the controls [131]. The same observation was made by Iturriza-Gomara and colaborators who identified more cases of rotavirus infections in children hospitalized for diarrheal diseases than in those from the community [132]. The highest proportion of rotavirus was observed in children aged 0-24 months and there was a statistically significant relationship between RVA infection and age group. This is similar to what has been reported elsewhere, as high levels of rotavirus are common during the first 24 months of life [133]. In addition, a high rate of RVA infections have been found in children under 7 months of age compared to other age groups. Our finding reinforces the need to further explore the role of exclusive breastfeeding.

Significant differences in terms of RVA proportions have been observed in this study when comparing diarrheal and non-diarrheal cases according to demographic data (age, gender, and location). This could be due to the difference in the number of participants between both groups. Although many other risk factors of rotavirus infections were previously identified [134], four different categorical variables (age, nutrition, source of drinking water, and diarrhea status) were identified as potential risk factors. This is concordant to what was reported by Fdhow et *al.,* highlighting that source of water was found to be a risk

factor for RVA infections [134]. Moreover, another study in line with our results has shown significant association between weaned children or those who had never been breastfed and RVA infections [135]. The important rate of RVA positive in children aged 0-6 months and the statistically significant relationship found between feeding practice and RVA infections supports the hypothesis that breastfeeding may not enhance protection against RVA infections [136]. Despite the small sample size of healthy children included, there was strong evidence confirming that the presence of rotavirus was related to diarrheal status. Overall, these findings concerning risk factors associated with RVA infections reinforce the need to improve the implementation of diarrhea control measures with emphasis on monitoring living conditions.

Regarding the circulating genotypes of RVA, G12 (25 %) was the most predominant followed by G1 and G8 (18 %), G3 (12 %), and G9 (2%). P[6] (31 %) and P[8] (38 %) were identified among RVA P-types. These results are similar to findings from neighbouring countries (Republic ofCongo and Cameroon), where most of the genotypes detected in our study were also reported [137,138]. P[8] (38 %) occurred as the most predominant P-genotype in this study, contrary to what was reported in a previous study in Gabon where P[6] (71.4 %) was the most detected followed by P[8] (23.8 %) [6]. This predominance of P[8] has been observed not only in Central Africa, but also in Central and South-Eastern Europe, [139]. However, a variable detection rate of RVA genotypes was observed over the study sites. For instance, our findings revealed that G1 (18 %) was lower than the proportion of G1 reported in Republic of Congo (57.5 %) and Central African Republic (37 %) [138,140].

Interestingly, our study reported a significant emergence of rotavirus G12 as well as the occurrence of G8 and G9 identified for the first time in Gabon, in addition to a bat-related G3 genotype. This could be explained by a possible animal source or by human migration into the region. In these regions where the population still lives in close contact with animals, potential interspecies or zoonotic transmission of group A rotavirus cannot be excluded [141].

Regarding G/P type combinations, we identified the G12P[6] combinations in 24 out of 121 samples (21 %) and the G1P[8] combinations in 19 out of 121 samples (17 %). This is in contrast to previous studies where G12P[6] was not reported [142] . The low proportion of G12P[8], as also reported previously, could be explained due to the fact that the RVA vaccine is not yet introduced in the country as the emergence of G12P[8] has been linked to vaccine pressure exerted on rotavirus types [142] .In addition, two rare genotype combinations G9P[8] and G8P[8] frequently detected in South-eastern Asia and some African countries were found in this study [143,144]. Multiple genotypes in the same host is a crucial factor for rotavirus reassortment, a process that creates new viral strains with differential adaptive and pathogenic potential.

The high percentage of non-typeable strains could suggest the circulation of viruses with unusual G and/or P genotypes that are not detected by the sets of primers adopted in our protocol. On the other hand, non-typeable strains may be strains of animal origin (P[1], P[5], P[7] and G5) that we did not specifically look for, or human strains that have undergone a mutation at the binding site of the primer specific to the genotype by genetic drift [139].

The phylogenetic analysis of RVA strains revealed a close relationship between Gabonese strains and those from East and South of Asia, Europe (Belgium) and other African countries. This reinforces what was mentioned above regarding the potential contribution of population movements in introducing RVA strains.

Amino acid changes at the antigenic epitopes on the VP7 and VP4 proteins of RVA can affect the capacity of antibodies to neutralize the virus. In this regard, alignment of the amino acid residues of the VP7 antigenic epitopes of Gabonese and vaccine strains were performed. G3 strain showed multiple amino acid changes at the antigenic epitopes on the VP7 strain compared to G1. An amino acid substitution (D97E) located in the 7-1a region was observed in comparison with the Rotateq G1 strain only, while one substitution was found at site 147 (N147D) compared to G1 strain from both vaccines. These mutations already reported in Iranian strains are important in virus neutralization [145].

Comparison of amino acid sequences of Gabonese G3 RVA strain and the G3 component of both vaccines indicated three changes at sites 242, 146, and 221. These amino acid residues do not belong to the site involved in neutralisation escape. When comparing this strain with the G3 Rotarix strain only, 9 residues were different out of 29 amino acid residues of these epitopes. In addition, one amino acid substitution (K238N) located in the 7-1b region was found by comparing Gabonese G3 RVA strain and the G3 component of Rota Teq. This mutation reported elsewhere, is responsible for a potential N-linked glycosylation site and could therefore affect the antigenicity of this epitope [146,147]. Interestingly, the bat transmitted G3 genotype identified in our study presented an amino acid substitution (K238D) by comparison with the G3 component of Rota Teq.

Since a previous study estimated that Rota Teq provides 83 % protection against G12 [70], a comparison of amino acid sequences of Gabonese G12 RVA strain (mostly detected in this study) as well as G8 strain and the corresponding components of both vaccines revealed multiple amino acid differences within the 3 VP7 trimers. This variability of amino acid at the antigenic epitopes on the VP7 was previously observed in Saint-Louis for G12 RVA strain [142].

The comparison of the VP8* fragment of VP4 of Gabonese P[8] strains with P[8] of both vaccine strains revealed a remarkable amount of differences. For P[8], which is in combination with G1 genotype, two substitutions were identified when compared with both vaccines and four changes were found when compared with Rotarix. Moreover, there were four differences when comparing P[8] from G8P[8] and G9P[8] RVA combinations and five substitutions when compared only with Rotarix. These findings are in line with other studies that have shown discrepancies in antigenic sites between wild-type and vaccine strains [146,147]. The P[8] strain of all RVA combinations found in our study showed the presence of an amino acid substitution at position 144 located in the 8-1 region compared to the two vaccine strains. This observation was different than what was reported previously [147].

Taken together, our findings offer baseline epidemiological and genetic data required for assessing the pre-vaccine epidemiological situation. It will be important to continue the research after the implementation of the vaccines in Gabon.

3.4. Diversity of other enteric viruses: norovirus, astrovirus, sapovirus and aichivirus

The diversity of enteric viruses is currently recognised as a threat to the development of preventive strategies, particularly vaccine development.

With this regard, Chapter 3 assessed the genetic diversity of enteric viruses in Gabonese children under five years of age. In this purpose, we screened four different enteric viruses, including NoV, SaV, HAstV and AiV among children with/without diarrhea, using PCR methods. Subsequently, phylogenetic analyses of different genome regions were performed to provide indication for recombinant strains, genetic diversity, and information about unusual strains of viruses causing diarrhea.

The detection rate of at least one viral agent was 23.2 % and 14.9 % for symptomatic and asymptomatic children, respectively. This number of enteric viruses' cases found in sick children is lower than what has been previously reported, whereas the detection rate in the control group was almost equal to the proportion reported by Zhang, SX et *al*., in 2016 [148,149]. This low proportion of enteric viruses found in symptomatic children could be explained by the fact that rotavirus known to be the most prevalent gastroenteritis virus and adenovirus were not screened in this study.

NoV (14.7 %) and HAstV (7.3 %) were the most detected enteric viruses in symptomatic children, while in asymptomatic children, NoV (9 %) and AiV (6 %) were found to be predominant. Our results are in concordance with previous studies, in which norovirus was identified as the most predominant after RVA and adenovirus, and HAstV ranked fourth among children with diarrhea [112,148]. In agreement with Ouédraogo et *al*., aichivirus detected for the first time in Gabon, was predominant in healthy children.

Overall, enteric viruses screened were found only in symptomatic children aged 0 to 24 months while for age group 25 to 59 months, highest infection rates were found in the controls. This higher frequency of viral infections in symptomatic children less than twenty-five months of age is in agreement with previous reports [150,151]. Moreover, this observation shows the highest susceptibility of the children to viral infections during early childhood, perhaps due to the fact that at this age, children crawl and are carried by people, thus are more likely to touch contaminated objects. The fact that most of the infection cases were found in the age group 25 to 59 months in asymptomatic children, while in sick children, they were identified during the two first years of life, could perhaps suggest that the acquisition of immunity against these infections occur after the age of 24 months [152].

Seasonal distribution throughout the sampling period was observed among children with diarrhea. NoV, SaV and HAstV were detected with major peaks during dry seasons, with NoV showing a significant seasonal pattern. These findings are in line with what was reported in Cameroon [153] and Nigeria [154]. In concordance with the previous observations, occurrence of AiV was observed during the rainy season [154,155].

Among norovirus positive cases, genogroup II (NoV GII) was the most detected in symptomatic children with a detection rate of 11.3%. This finding is consistent with the study which showed that NoV GII plays a major role in sporadic diarrheal diseases [156]. Moreover, the data presented in this study shows the predominance of capsid genotype GII.4 and polymerase genotype GII.P31. As observed elsewhere, the emergence or the predominance of GII.4 could perhaps be explained by the increase in tropism or affinity of norovirus for histo-blood group antigens [157]. Predominance in GII.31 (formerly known as the GII.Pe) reinforces speculation pointing out that GII.P31 has obviously replaced GII.P4 as the leading RdRp genotype in children with sporadic gastroenteritis [158]. Furthermore, GII.Pe/GII4 Sydney (newly named GII.P31-GII.4 Sydney) firstly reported in Australia in 2012, subsequently widespread in South Africa (2012-2013), Iran (2015-2016), Korea (2013) and China (2012-2015), was also found in this report with a proportion of 45.2 % [159–164]. These two genotypes GII.P31

et GII.4 Sydney detected in our study were closely related to the Australian reference strains identified in 2012 (with the GenBank accession numbers JX459907 and JX459908 respectively). While four GII.P17 strains were closer to Japanese strains (LC037415) and the three capsid genotypes GII.17 were closer to the American strain identified in 2016 (AY502009). These results revealed that the same strains are circulating across the world and population movements facilitate the importation of global NoV strains [165].

Our results are similar to findings of Lekana's study, in which human astrovirus was identified as the third pathogen after NoV and SaV [112]. The detection rate of HAstV found in children with diarrhea is relatively similar to what was reported in Nigeria (6.8 %) and Burkina Faso (4.9 %) [148,166]. In contrast to previous studies from France, China, and Spain that reported HAstV1 as the predominant strain, ours did not find this genotype in the study population [167,168]. However, HAstV5 and HAstV4 were mostly detected in children with diarrhea. Our finding reported the presence of the highly divergent human astrovirus MLB1 strain in one symptomatic child. Moreover, three cases of novel astrovirus strains (Ast-VA1) known to be associated with AGE outbreak was found in our study population [169]..

Human sapovirus was detected in 3.4 % of the cases and 1.5 % in healthy children. This finding was similar to previous studies that reported a lower detection rate of SaV than NoV. [148,170]. Genogroup I SaV was predominant among all SaV positive samples genotyped. In concordance with Audrey Cilli et *al.*, 2019, SaV-GI.2 and SaV -GI.3 was identified in children with diarrhea [171], while SaV-GI.1 was found in one symptomatic case as well as in one asymptomatic child. Although less frequent in this present study than in Burkinabe children with diarrhea [148], one case of GII.4 has been characterized among SaV-GII.

In agreement with previous findings, our study reported a low proportion of AiV (2.5 %) in the study population, with more cases in asymptomatic participants [172]. This could reinforce the fact that the clinical role of AiV as gastroenteritis agent is still unclear. Contrary to previous findings of the presence of AiV A in

children with gastroenteritis [173,174], the AiV A detected in our study came from healthy children. This reinforces the need for further studies to elucidate the full spectrum of clinical symptoms of AiV. The rare AiV C reported only in Burkina Faso [148] and France [175] in children with gastroenteritis was also identified in one symptomatic child in our study. Our study was the first to report AiV in Central Africa, including the rare AiV genotype C, already reported only in France and Burkina Faso [95,148].

In conclusion, diarrheal diseases remain a major public health issue in developing countries. There are over 2.5 billion diarrheal illnesses cases annually worldwide and an estimated 1,400 deaths daily. Current diarrhea control programs are failing in some regions due to lack of awareness, routine diagnosis, and effective epidemiological surveillance to sustain and enhance diarrhea control. However, documenting the aetiology of diarrhea is important to guide vaccine development, design prevention and treatment strategies. In this work, a spectrum of infectious agents found in children with diarrhea was reported. These findings can serve as baseline data for public health stakeholders and guide more detailed future studies. The findings of the second publication demonstrate that CerTest Crypto RDT has low sensitivity for the detection of *Cryptosporidium spp*. Thus, this rapid diagnostic test should not be used as an appropriate tool to guide community-based screening programs. The findings of the third and fourth manuscripts show a great diversity and emerging enteric virus strains in the Gabonese population. The third study provides insight into the circulating rotavirus genotypes prior to vaccine introduction in the country and reveals amino acid substitutions in the G and P antigenic epitopes, which could influence vaccine efficacy. Overall, this dissertation promotes prompt information refinement of diarrheal control and elimination strategies.

Although the data does not directly reflect the scenario related to the causality of diarrhea, our reports suggest active surveillance of circulating enteropathogens in the community. In addition, important information was highlighted on the burden of RVA in Gabon and on the diagnosis of *Cryptosporidium*. Several studies could now be conducted to help extend this work. These may include:

-The pattern of rotavirus transmission in the community. Our study reported the presence of a bat-related RVA G3 genotype in a symptomatic child. This finding raised the question of the transmission route of RVA strains in the community and frequence of zoonotic transmission

- Post-vaccination rotavirus surveillance. Chapter 4 showed the presence of RVA in unvaccinated, symptomatic, and asymptomatic children. The G1P[8] genotype combination targeted by both vaccines was the second most prevalent strain.

- Development of nucleic acid-based rapid diagnostic test. As detailed in chapter 2, this issue was raised by the performance of the immunochromatographic test used for the detection of *Cryptosporidium* among African children.

4. SUMMARY

Diarrheal diseases collectively constitute a serious public health challenge globally. The causative agents of diarrheal disease include adenovirus (serotypes 40 and 41), *Aeromonas* spp, *Entamoeba histolytica*, *Cryptosporidium spp.*, *Escherichia coli* strains, norovirus (NoV), non-typhoidal *Salmonella spp.*, rotavirus A (RVA), *Shigella spp*., *Vibrio cholerae* and *Clostridium difficile.* Among these, rotavirus, *Cryptosporidium spp*., and *Shigella spp.* are the three aetiological agents responsible for most deaths in children under 5 years old. The distributions of these pathogens are overlapping and they coexist in many endemic areas, particularly in low-income countries. Accurate diagnosis and molecular characterization of diarrheal pathogens are necessary for surveillance, prevention, and control of diarrhea. To obtain accurate epidemiological data,to support diarrheal disease control and elimination intervention strategies in Gabon, four studies were conducted as part of this thesis.

First, the prevalence of pathogens found in stool samples in outpatient Gabonese children < 5 years with diarrhea was for the first time systematically investigated to describe the local spectrum of infectious agents. The most frequently identified were *enteroinvasive Escherichia coli* (EIEC) /*Shigella* and *Enterotoxigenic Escherichia coli* (ETEC), followed by *Giardia lamblia*, *Cryptosporidium spp.* and rotavirus. The emergent ETEC, EIEC /*Shigella*, *Cryptosporidium spp.* and rotavirus were frequently detected in combination. The most frequently observed combinations of pathogens were EIEC/*Shigella* and ETEC, ETEC and rotavirus as well as *Cryptosporidium* and EIEC/*Shigella*. This information serves as baseline for recommendation for interventions and diagnostic algorithms to public health stakeholders.

The second study was nested in a broader community-based project and aimed to evaluate cryptosporidiosis diagnosis by a rapid diagnostic test (CerTest Crypto RDT) against a composite reference of quantitative polymerase chain reaction (qPCR) and restriction fragment length polymorphism (RFLP)-PCR in African children from four countries (Gabon, Ghana, Madagascar, and Tanzania) admitted to a hospital with diarrhea. The performance of this RDT varied across

the four study sites. Overall, the test showed a low sensitivity for the detection of *Cryptosporidium* parvum and *C*. *hominis*.

This work included the comprehensive investigation of rotavirus genotypes and antigenic epitope variability in the VP7 and VP4 proteins of circulating rotavirus A strains compared to vaccine strains, as the third part of this dissertation. Rotavirus A was detected in 55 % (98/177) of hospitalized children with gastroenteritis and 21 % (14/67) of the control children. The most common genotypes were G1, G3, G8, G9, G12, with G8 and G9 being reported for the first time in Gabon. All of these G genotypes were associated either with P[6] or P[8] genotypes. Several amino acid mutations associated with immune evasion were detected on antigenic epitopes of VP7 (sites 94, 147) and VP8* (sites 89, 116, 146, 150) of Gabonese strains, which may lead to reduced efficacy of available RotaTeq and Rotarix vaccines.

The fourth study was designed to determine the prevalence and genetic diversity of four main enteric viruses (*Norovirus*, *Sapovirus*, *Astrovirus* and *Aichvirus* A) in hospitalized children <5 years with gastroenteritis and community controls without gastroenteritis. Norovirus (14.7 %; 26/177) and astrovirus (7.3 %; 13/177) were the most prevalent in children with diarrhea, while in the healthy group norovirus (9 %; 6/67) followed by aichivirus A (6 %; 4/67) were predominant. The predominant norovirus genogroup was GII, consisting mostly of genotype GII.P31-GII.4 Sydney. This study provides the first report on the detection of Aichvirus A in Gabon and Central Africa.

This thesis provides the epidemiological and genetic baseline data that will be essential for advocating a much-needed management of diarrhea as recommended by the WHO. Moreover, these results show that the implementation of a national vaccination program against rotavirus is necessary and urgent in Gabon. The findings related to the performance of CerTest Crypto RDT in Africa will support implementation of diarrhea control and elimination efforts in endemic areas. Future work should focus on the development of laboratory methods to improve the sensitivity of cryptosporidiosis detection and on expanding the use of routine diagnosis.

5. GERMAN SUMMARY (ZUSAMMENFASSUNG)

Durchfallerkrankungen stellen zusammengenommen weltweit eine ernsthafte Herausforderung für die öffentliche Gesundheit dar. Zu den Erregern von Durchfallerkrankungen gehören Adenovirus (Serotypen 40 und 41), *Aeromonas spp*., *Entamoeba histolytica*, *Cryptosporidium spp*., *Escherichia coli*-Stämme, Norovirus (NoV), nicht-typhoidale *Salmonella spp*., rotavirus A (RVA), *Shigella spp*., *Vibrio cholerae* und *Clostridium difficile*. Unter diesen sind Rotavirus, *Cryptosporidium spp*. und *Shigella spp*. die drei Ätiologien, die für die meisten Todesfälle bei Kindern unter 5 Jahren verantwortlich sind. Die Verbreitungen dieser Krankheitserreger überschneiden sich und sie koexistieren in vielen Endemiegebieten, insbesondere in Ländern mit niedrigem Einkommen. Eine genaue Diagnose und molekulare Charakterisierung von Durchfallerregern ist für die Überwachung, Prävention und Kontrolle von Durchfall erforderlich. Um genaue epidemiologische Daten zu erhalten, um die Bekämpfung von Durchfallerkrankungen und Interventionsstrategien zur Eliminierung in Gabun zu unterstützen, wurden im Rahmen dieser Arbeit vier Studien durchgeführt.

Zunächst wurde die Prävalenz von Erregern in Stuhlproben ambulanter gabunischer Kinder < 5 Jahre mit Durchfall erstmals systematisch untersucht, um das lokale Erregerspektrum zu beschreiben. Am häufigsten wurden enteroinvasive *Escherichia coli* (EIEC) /*Shigella* und enterotoxigene *Escherichia coli* (ETEC) identifiziert, gefolgt von *Giardia lamblia*, *Cryptosporidium spp*. und Rotaviren. Die aufstrebenden ETEC, EIEC /*Shigella*, *Cryptosporidium spp*. und Rotavirus wurden häufig in Kombination nachgewiesen. Die am häufigsten beobachteten Erregerkombinationen waren EIEC/*Shigella* und ETEC, ETEC und Rotavirus sowie *Cryptosporidium* und EIEC/*Shigella*. Diese Informationen dienen als Grundlage für Empfehlungen für Interventionen und Diagnosealgorithmen an Interessenvertreter des öffentlichen Gesundheitswesens.

Die zweite Studie war in ein breiter angelegtes Gemeinschaftsprojekt eingebettet und zielte darauf ab, die Kryptosporidiose-Diagnose durch einen diagnostischen Schnelltest (CerTest Crypto RDT) anhand einer zusammengesetzten Referenz aus quantitativer Polymerase-Kettenreaktion (qPCR) und

Restriktionsfragmentlängen-Polymorphismus (RFLP)-PCR zu bewerten bei afrikanischen Kindern aus vier Ländern (Gabun, Ghana, Madagaskar und Tansania), die mit Durchfall ins Krankenhaus eingeliefert wurden. Die Leistung dieses RDT variierte zwischen den vier Studienzentren. Insgesamt zeigte der Test eine geringe Sensitivität für den Nachweis von *Cryptosporidium parvum* und *Cryptosporidium hominis*.

Diese Arbeit beinhaltete eine umfassende Untersuchung von Rotavirus Genotypen und antigene Epitopvariabilität in den VP7- und VP4-Proteinen von zirkulierenden Rotavirus-A-Stämmen im Vergleich zu Impfstämmen als dritten Teil dieser Dissertation Rotavirus A wurde bei 55 % (98/177) der hospitalisierten Kinder mit Gastroenteritis und bei 21 % (14/67) der Kontrollen nachgewiesen. Die häufigsten Genotypen waren G1, G3, G8, G9, G12, wobei G8 und G9 zum ersten Mal in Gabun beschrieben wurden.

Alle diese G-Genotypen waren entweder mit P[6]- oder P[8]-Genotypen assoziiert. Auf antigenen Epitopen von VP7 (Stellen 94, 147) und VP8* (Stellen 89, 116, 146, 150) von gabunischen Stämmen wurden mehrere Aminosäuremutationen im Zusammenhang mit Immunevasion nachgewiesen, was zu einer verringerten Wirksamkeit der verfügbaren RotaTeq- und Rotarix-Impfstoffe führen könnte.

Die vierte Studie wurde entwickelt, um die Prävalenz und genetische Vielfalt der vier wichtigsten enterischen Viren (*Norovirus*, *Sapovirus*, *Astrovirus* und *Aichvirus* A) bei hospitalisierten Kindern unter 5 Jahren mit Gastroenteritis und ambulanten Kontrollen ohne Gastroenteritis zu bestimmen. Norovirus (14,7 %; 26/177) und Astrovirus (7,3 %; 13/177) waren bei Kindern mit Durchfall am häufigsten, während in der gesunden Gruppe Norovirus (9 %; 6/67) gefolgt von Aichivirus A (6 %; 4/67) dominierten. Die vorherrschende Norovirus-Genogruppe war GII, die hauptsächlich aus dem Genotyp GII.P31-GII.4 Sydney bestand. Diese Studie liefert den ersten Bericht über den Nachweis von Aichvirus A in Gabun und Zentralafrika.

Diese Dissertation liefert die epidemiologischen und genetischen Basisdaten, die für die Prävention und Kontrolle von Durchfallerkrankungen, wie von der WHO

empfohlen, unerlässlich sind. Darüber hinaus machen diese Ergebnisse die Einführung der Impfung gegen Rotavirus in das nationale Impfprogramm notwendig und dringend. Die Erkenntnisse zum Verständnis der Leistung von CerTest Crypto RDT in Afrika werden die Umsetzung von Durchfallbekämpfungs- und Eliminierungsbemühungen in Endemiegebieten unterstützen. Zukünftige Arbeiten sollten sich auf die Entwicklung von Labormethoden zur Verbesserung der Sensitivität des Kryptosporidiose-Nachweises und auf die Ausweitung des Einsatzes der Routinediagnostik konzentrieren.

6. BIBLIOGRAPHY

1. Kotloff, K.L.; Nataro, J.P.; Blackwelder, W.C.; Nasrin, D.; Farag, T.H.; Panchalingam, S.; Wu, Y.; Sow, S.O.; Sur, D.; Breiman, R.F.; et al. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): A prospective, case-control study. *Lancet* **2013**, *382*, 209–222, doi:10.1016/S0140-6736(13)60844-2.

2. Troeger, C.; Forouzanfar, M.; Rao, P.C.; Khalil, I.; Brown, A.; Reiner, R.C.; Fullman, N.; Thompson, R.L.; Abajobir, A.; Ahmed, M.; et al. Estimates of global, regional, and national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet Infect. Dis.* **2017**, *17*, 909–948, doi:10.1016/S1473-3099(17)30276-1.

3. Liu, L.; Oza, S.; Hogan, D.; Chu, Y.; Perin, J.; Zhu, J.; Lawn, J.E.; Cousens, S.; Mathers, C.; Black, R.E. Global, regional, and national causes of under-5 mortality in 2000–15: an updated systematic analysis with implications for the Sustainable Development Goals. *Lancet* **2016**, *388*, 3027–3035, doi:10.1016/S0140-6736(16)31593-8.

4. United Nations Transforming our world: the 2030 Agenda for Sustainable Development.

5. Koko, J.; Ambara, J.-P.; Ategbo, S.; Gahouma, D. Épidémiologie des diarrhées aiguës bactériennes de l'enfant à Libreville, Gabon. *Arch. Pédiatrie* **2013**, *20*, 432–433, doi:10.1016/j.arcped.2013.01.017.

6. Lekana-Douki, S.E.; Kombila-Koumavor, C.; Nkoghe, D.; Drosten, C.; Drexler, J.F.; Leroy, E.M. Molecular epidemiology of enteric viruses and genotyping of rotavirus A, adenovirus and astrovirus among children under 5 years old in Gabon. *Int. J. Infect. Dis.* **2015**, *34*, 90–95, doi:10.1016/j.ijid.2015.03.009.

7. Oyegue-Liabagui, S.L.; Ndjangangoye, N.K.; Kouna, L.C.; Lekolo, G.M.; Mounioko, F.; Kwedi Nolna, S.; Lekana-Douki, J.B. Molecular prevalence of intestinal parasites infections in children with diarrhea in Franceville, Southeast of Gabon. *BMC Infect. Dis.* **2020**, *20*, 350, doi:10.1186/s12879-020-05071-x.

8. Duong, T.H.; Kombila, M.; Dufillot, D.; Richard-Lenoble, D.; Owono Medang, M.; Martz, M.; Gendrel, D.; Engohan, E.; Moreno, J. Role of cryptosporidiosis in infants in Gabon. Results of two prospective studies. *Bull Soc Pathol Exot* **1991**, *84*.

9. Duong, T.H.; Dufillot, D.; Koko, J.; Nze-Eyo'o, R.; Thuilliez, V.; Richard-Lenoble, D.; Kombila, M.; Owono-Medang, avec la collaboration technique de Mathieu Owono-Medang. Cryptosporidiose digestive chez le jeune enfant en zone urbaine au Gabon. *Cah. d'études Rech. Francoph. / Santé* **1995**, *5*, 185–188.

10. Troeger, C.; Forouzanfar, M.; Rao, P.C.; Khalil, I.; Brown, A.; Reiner, R.C.; Fullman, N.; Thompson, R.L.; Abajobir, A.; Ahmed, M.; et al. Estimates of global, regional, and national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet Infect. Dis.* **2017**, *17*, 909–948, doi:10.1016/S1473-3099(17)30276-1.

11. Ahs, J.; Tao, W.; Löfgren, J.; Forsberg, B.C.; Albert, M.J.; Faruque, A.S.G.; Faruque, S.M.; Sack, R.B.; Mahalanabis, D.; Awasthi, S.; et al. Why children are still dying and what can be done; **2004**; Vol. 11; ISBN 9789280644623.

12. Kim, Y.J.; Park, K.H.; Park, D.A.; Park, J.; Bang, B.W.; Lee, S.S.; Lee, E.J.; Lee, H.J.; Hong, S.K.; Kim, Y.R. Guideline for the antibiotic use in acute gastroenteritis. *Infect. Chemother.* **2019**, *51*, 217–243, doi:10.3947/ic.2019.51.2.217.

13. Centers for Disease Control and Prevention Global Diarrhea Burden Diarrhea: Common Illness, Global Killer. *U.S. Dep. Heal. Hum. Serv.* **2013**, 1–4.

14. IVAC. Pneumonia and diarrhea progress report. *Int. Vaccine Access Cent.* **2014**, 1–12.

15. Wardlaw, T.; Salama, P.; Brocklehurst, C.; Chopra, M.; Mason, E. Diarrhoea: why children are still dying and what can be done. *Lancet* **2010**, *375*, 870–872, doi:10.1016/S0140-6736(09)61798-0.

16. Dupont, C. Diarrhées aiguës de l'enfant. *J. Pediatr. Pueric.* **2010**, *23*, 84–95, doi:10.1016/j.jpp.2010.03.008.

17. Lorrot, M.; Vasseur, M. Physiopathology of rotavirus diarrhea. *J. Pédiatrie Puériculture* **2007**, *20*, 330–336, doi:10.1016/j.jpp.2007.11.010.

18. Bouseettine, R.; Hassou, N.; Bessi, H.; Ennaji, M.M. Waterborne transmission of enteric viruses and their impact on public health; Elsevier Inc., **2019**; ISBN 9780128194003.

19. Troeger, C.; Blacker, B.F.; Khalil, I.A.; Rao, P.C.; Cao, S.; Zimsen, S.R.; Albertson, S.B.; Stanaway, J.D.; Deshpande, A.; Abebe, Z.; et al. Estimates of the global, regional, and national morbidity, mortality, and aetiologies of diarrhoea in 195 countries: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Infect. Dis.* **2018**, *18*, 1211–1228, doi:10.1016/S1473-3099(18)30362-1.

20. Steele, A.D.; Glass, R. Rotavirus in South Africa: From discovery to vaccine introduction. *South. African J. Epidemiol. Infect.* **2011**, *26*, 184–190, doi:10.1080/10158782.2011.11441448.

21. Lin, C.-L.; Chen, S.-C.; Liu, S.-Y.; Chen, K.-T. Disease Caused by Rotavirus Infection. *Open Virol. J.* **2014**, *8*, 14–19, doi:10.2174/1874357901408010014.

22. Lanzieri, T.M.; Linhares, A.C.; Costa, I.; Kolhe, D.A.; Cunha, M.H.; Ortega-Barria, E.; Colindres, R.E. Impact of rotavirus vaccination on childhood deaths from diarrhea in Brazil. *Int. J. Infect. Dis.* **2011**, *15*, e206–e210, doi:10.1016/j.ijid.2010.11.007.

23. Gastañaduy, P.A.; Steenhoff, A.P.; Mokomane, M.; Esona, M.D.; Bowen, M.D.; Jibril, H.; Pernica, J.M.; Mazhani, L.; Smieja, M.; Tate, J.E.; et al. Effectiveness of Monovalent Rotavirus Vaccine after Programmatic Implementation in Botswana: A Multisite Prospective Case-Control Study. *Clin. Infect. Dis.* **2016**, *62*, S161–S167, doi:10.1093/cid/civ1207.

24. Groome, M.J.; Page, N.; Cortese, M.M.; Moyes, J.; Zar, H.J.; Kapongo, C.N.; Mulligan, C.; Diedericks, R.; Cohen, C.; Fleming, J.A.; et al. Effectiveness of monovalent human rotavirus vaccine against admission to hospital for acute rotavirus diarrhoea in South African children: A case-control study. *Lancet Infect. Dis.* **2014**, *14*, 1096–1104, doi:10.1016/S1473-3099(14)70940-5.

25. Checkley, W.; White, A.C.; Jaganath, D.; Arrowood, M.J.; Chalmers, R.M.; Chen, X.-M.; Fayer, R.; Griffiths, J.K.; Guerrant, R.L.; Hedstrom, L.; et al. A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for cryptosporidium. *Lancet. Infect. Dis.* **2015**, *15*, 85–94, doi:10.1016/S1473-3099(14)70772-8.

26. Scallan, E.; Hoekstra, R.M.; Angulo, F.J.; Tauxe, R. V.; Widdowson, M.A.; Roy, S.L.; Jones, J.L.; Griffin, P.M. Foodborne illness acquired in the United States-Major pathogens. *Emerg. Infect. Dis.* **2011**, *17*, 7–15, doi:10.3201/eid1701.P11101.

27. Womack, N.C. Treatment of diarrhea. *South. Med. J.* **1924**, *17*, 562–563, doi:10.1097/00007611-192408000-00004.

28. WHO Diarrhoeal disease. **2017**.

29. Tyzzer, E.E. A sporozoon found in the peptic glands of the common mouse. *Soc. Exp. Biol. Med.* **1907**, *5*, 12–13.

30. Tyzzer, E. An extracellular coccidium, Cryptosporidium muris (gen. & sp. nov.), of the gastric glands of the common mouse. *J. Med. Res.* **1910**, *18*, 487–509.

31. Clode, P.L.; Koh, W.H.; Thompson, R.C.A. Life without a Host Cell: What is Cryptosporidium? *Trends Parasitol.* **2015**, *31*, 614–624, doi:10.1016/j.pt.2015.08.005.

32. Alvarez-Pellitero, P.; Quiroga, M.I.; Sitjà-Bobadilla, A.; Redondo, M.J.; Palenzuela, O.; Padrós, F.; Vázquez, S.; Nieto, J.M. Cryptosporidium scophthalmi n. sp. (Apicomplexa: Cryptosporidiidae) from cultured turbot Scophthalmus maximus. Light and electron microscope description and histopathological study. *Dis. Aquat. Organ.* **2004**, *62*, 133–145, doi:10.3354/dao062133.

33. Ren, X.; Zhao, J.; Zhang, L.; Ning, C.; Jian, F.; Wang, R.; Lv, C.; Wang, Q.;

Arrowood, M.J.; Xiao, L. Cryptosporidium tyzzeri n. sp. (Apicomplexa: Cryptosporidiidae) in domestic mice (Mus musculus). *Exp. Parasitol.* **2012**, *130*, 274–281, doi:10.1016/j.exppara.2011.07.012.

34. Ryan, U.; Paparini, A.; Tong, K.; Yang, R.; Gibson-Kueh, S.; O'Hara, A.; Lymbery, A.; Xiao, L. Cryptosporidium huwi n. sp. (Apicomplexa: Eimeriidae) from the guppy (Poecilia reticulata). *Exp. Parasitol.* **2015**, *150*, 31–35, doi:10.1016/j.exppara.2015.01.009.

35. Fayer, R.; Ungar, B. Cryptosporidium Cryptosporidiosis. *Microbiol. Rev.* **1986**, *50*, 458–483.

36. Ryan, U.; Fayer, R.; Xiao, L. Cryptosporidium species in humans and animals: Current understanding and research needs. *Parasitology* **2014**, *141*, 1667–1685, doi:10.1017/S0031182014001085.

37. Šlapeta, J. Cryptosporidiosis and Cryptosporidium species in animals and humans: A thirty colour rainbow? *Int. J. Parasitol.* **2013**, *43*, 957–970, doi:10.1016/j.ijpara.2013.07.005.

38. Ayinmode, A.B.; Oliveira, B.C.M.; Obebe, O.O.; Dada-Adgebola, H.O.; Ayede, A.I.; Widmer, G. Genotypic characterization of cryptosporidium species in humans and peri-domestic animals in Ekiti and Oyo States, Nigeria. *J. Parasitol.* **2018**, *104*, 718–721, doi:10.1645/17-74.

39. Shrivastava, A. K., Kumar, S., Smith, W. A., & Sahu, P.S. Revisiting the global problem of cryptosporidiosis and recommendations. *Trop. Parasitol.* **2017**.

40. Dong, S.; Yang, Y.; Wang, Y.; Yang, D.; Yang, Y.; Shi, Y.; Li, C.; Li, L.; Chen, Y.; Jiang, Q.; et al. Prevalence of Cryptosporidium Infection in the Global Population: A Systematic Review and Meta-analysis. *Acta Parasitol.* **2020**, *65*, 882–889, doi:10.2478/s11686-020-00230-1.

41. Bouzid, M.; Kintz, E.; Hunter, P.R. Risk factors for Cryptosporidium infection in low and middle income countries: A systematic review and meta-analysis. *PLoS Negl. Trop. Dis.* **2018**, *12*, 1–13, doi:10.1371/journal.pntd.0006553.

42. Shoultz, D.A.; de Hostos, E.L.; Choy, R.K.M. Addressing Cryptosporidium Infection among Young Children in Low-Income Settings: The Crucial Role of New and Existing Drugs for Reducing Morbidity and Mortality. *PLoS Negl. Trop. Dis.* **2016**, *10*, 1–7, doi:10.1371/journal.pntd.0004242.

43. Hunter, P.R. Water-borne disease: epidemiology and ecology. *Microbiol. Virol.* **1997**.

44. Gerace, E.; Presti, V.D.M. Lo; Biondo, C. Cryptosporidium infection: epidemiology, pathogenesis, and differential diagnosis . *Eur. J. Microbiol. Immunol.* **2019**, *9*, 119–123, doi:10.1556/1886.2019.00019.

45. Chalmers, R.M.; Davies, A.P. Minireview: Clinical cryptosporidiosis. *Exp. Parasitol.* **2010**, *124*, 138–146, doi:10.1016/j.exppara.2009.02.003.

46. Mondal, D.; Minak, J.; Alam, M.; Liu, Y.; Dai, J.; Korpe, P.; Liu, L.; Haque, R.; Petri, W.A. Contribution of enteric infection, altered intestinal barrier function, and maternal malnutrition to infant malnutrition in Bangladesh. *Clin. Infect. Dis.* **2012**, *54*, 185–192, doi:10.1093/cid/cir807.

47. Hunter, P.R.; Nichols, G. Epidemiology and clinical features of Cryptosporidium infection in immunocompromised patients. *Clin. Microbiol. Rev.* **2002**, *15*, 145–154, doi:10.1128/CMR.15.1.145-154.2002.

48. Wang, R. jun; Li, J. qiang; Chen, Y. cai; Zhang, L. xian; Xiao, L. hua Widespread occurrence of Cryptosporidium infections in patients with HIV/AIDS: Epidemiology, clinical feature, diagnosis, and therapy. *Acta Trop.* **2018**, *187*, 257–263, doi:10.1016/j.actatropica.2018.08.018.

49. Bailey, J.M.; Erramouspe, J. Nitazoxanide Treatment for Giardiasis and Cryptosporidiosis in Children. *Ann. Pharmacother.* **2004**, *38*, 634–640, doi:10.1345/aph.1D451.

50. Ii, A.; Sh, A.; Arumugam, C.; Pr, H.; Usman, N. Cochrane Database of Systematic Reviews Prevention and treatment of cryptosporidiosis in immunocompromised patients (Review) Prevention and treatment of cryptosporidiosis in immunocompromised patients (Review). **2012**, doi:10.1002/14651858.CD004932.pub2.www.cochranelibrary.com.

51. Crawford, S.E.; Ramani, S.; Tate, J.E.; Parashar, U.D.; Svensson, L.; Hagbom, M.; Franco, M.A.; Greenberg, H.B.; O'ryan, M.; Kang, G.; et al. Rotavirus infection HHS Public Access. *JAMA Pediatr.* **2018**, *172*, 50–53, doi:10.1038/nrdp.2017.83.Rotavirus.

52. Esona, M.D.; Gautam, R. Rotavirus. *Clin. Lab. Med.* **2015**, *35*, 363–391, doi:10.1016/j.cll.2015.02.012.

53. Van Doorn, L.J.; Kleter, B.; Hoefnagel, E.; Stainier, I.; Poliszczak, A.; Colau, B.; Quint, W. Detection and genotyping of human rotavirus VP4 and VP7 genes by reverse transcriptase PCR and reverse hybridization. *J. Clin. Microbiol.* **2009**, *47*, 2704–2712, doi:10.1128/JCM.00378-09.

54. Matthijnssens, J.; Ciarlet, M.; Heiman, E.; Arijs, I.; Delbeke, T.; McDonald, S.M.; Palombo, E.A.; Iturriza-Gómara, M.; Maes, P.; Patton, J.T.; et al. Full Genome-Based Classification of Rotaviruses Reveals a Common Origin between Human Wa-Like and Porcine Rotavirus Strains and Human DS-1-Like and Bovine Rotavirus Strains. *J. Virol.* **2008**, *82*, 3204–3219, doi:10.1128/jvi.02257-07.

55. Cortese, M.M.; Parashar, U.D. Prevention of Rotavirus Gastroenteritis Among Infants and Children Recommendations of the Advisory Committee. *MMWR Recomm Reports* **2009**, *58*, 1–25.

56. Desselberger, U. Rotaviruses. *Virus Res.* **2014**, *190*, 75–96, doi:10.1016/j.virusres.2014.06.016.

57. de Waure, C.; Sarnari, L.; Chiavarini, M.; Ianiro, G.; Monini, M.; Alunno, A.; Camilloni, B. 10-year rotavirus infection surveillance: Epidemiological

trends in the pediatric population of Perugia province. *Int. J. Environ. Res. Public Health* **2020**, *17*, 1–9, doi:10.3390/ijerph17031008.

58. Troeger, C.; Khalil, I.A.; Rao, P.C.; Cao, S.; Blacker, B.F.; Ahmed, T.; Armah, G.; Bines, J.E.; Brewer, T.G.; Colombara, D. V.; et al. Rotavirus Vaccination and the Global Burden of Rotavirus Diarrhea among Children Younger Than 5 Years. *JAMA Pediatr.* **2018**, *172*, 958–965, doi:10.1001/jamapediatrics.2018.1960.

59. Santos, N.; Hoshino, Y. Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine. *Rev. Med. Virol.* **2005**, *15*, 29–56, doi:10.1002/rmv.448.

60. Kirkwood, C.D. Genetic and antigenic diversity of human rotaviruses: Potential impact on vaccination programs. *J. Infect. Dis.* **2010**, *202*, 43–48, doi:10.1086/653548.

61. Cunliffe, Nigel , Joseph S Bresee , Jon R Gentsch , Roger, C.A.H. The expanding diversity of rotaviruses For personal use . Only reproduce with permission from The Lancet Publishing Group . *Lancet* **2002**, *359*, 640–642.

62. Gentsch, J.R.; Laird, A.R.; Bielfelt, B.; Griffin, D.D.; Bányai, K.; Ramachandran, M.; Jain, V.; Cunliffe, N.A.; Nakagomi, O.; Kirkwood, C.D.; et al. Serotype diversity and reassortment between human and animal rotavirus strains: Implications for rotavirus vaccine programs. *J. Infect. Dis.* **2005**, *192*, doi:10.1086/431499.

63. Daniel C. Payne, PhD, MSPH; Umesh D. Parashar, MBBS, M. VPD Surveillance Manual Rotavirus: Chapter 13.5. *Cdc* **2014**.

64. Grimwood, K.; Lambert, S.B.; Milne, R.J. Rotavirus infections and vaccines: Burden of illness and potential impact of vaccination. *Pediatr. Drugs* **2010**, *12*, 235–256, doi:10.2165/11537200-000000000-00000.

65. Bibera, G.L.; Chen, J.; Pereira, P.; Benninghoff, B. Dynamics of G2P[4] strain evolution and rotavirus vaccination: A review of evidence for Rotarix. *Vaccine* **2020**, *38*, 5591–5600, doi:10.1016/j.vaccine.2020.06.059.

66. Patel, M.M.; Parashar, U.D. Assessing the effectiveness and public health impact of rotavirus vaccines after introduction in immunization programs. *J. Infect. Dis.* **2009**, *200*, doi:10.1086/605059.

67. Boom, J.A.; Tate, J.E.; Sahni, L.C.; Rench, M.A.; Quaye, O.; Mijatovic-Rustempasic, S.; Patel, M.M.; Baker, C.J.; Parashar, U.D. Sustained protection from pentavalent rotavirus vaccination during the second year of life at a large, Urban United States Pediatric Hospital. *Pediatr. Infect. Dis. J.* **2010**, *29*, 1133–1135, doi:10.1097/INF.0b013e3181ed18ab.

68. Anderson, E.J.; Shippee, D.B.; Weinrobe, M.H.; Davila, M.D.; Katz, B.Z.; Reddy, S.; Cuyugan, M.G.K.P.; Lee, S.Y.; Simons, Y.M.; Yogev, R.; et al. Indirect protection of adults from rotavirus by pediatric rotavirus

vaccination. *Clin. Infect. Dis.* **2013**, *56*, 755–760, doi:10.1093/cid/cis1010.

69. Leshem, E.; Moritz, R.E.; Curns, A.T.; Zhou, F.; Tate, J.E.; Lopman, B.A.; Parashar, U.D. Rotavirus vaccines and health care utilization for diarrhea in the United States (2007-2011). *Pediatrics* **2014**, *134*, 15–23, doi:10.1542/peds.2013-3849.

70. Payne, D.C.; Boom, J.A.; Staat, M.A.; Edwards, K.M.; Szilagyi, P.G.; Klein, E.J.; Selvarangan, R.; Azimi, P.H.; Harrison, C.; Moffatt, M.; et al. Effectiveness of pentavalent and monovalent rotavirus vaccines in concurrent use among US children <5 years of age, 2009-2011. *Clin. Infect. Dis.* **2013**, *57*, 13–20, doi:10.1093/cid/cit164.

71. Armah, G.E.; Sow, S.O.; Breiman, R.F.; Dallas, M.J.; Tapia, M.D.; Feikin, D.R.; Binka, F.N.; Steele, A.D.; Laserson, K.F.; Ansah, N.A.; et al. Efficacy of pentavalent rotavirus vaccine against severe rotavirus gastroenteritis in infants in developing countries in sub-Saharan Africa: A randomised, double-blind, placebo-controlled trial. *Lancet* **2010**, *376*, 606–614, doi:10.1016/S0140-6736(10)60889-6.

72. Madhi, S.A.; Cunliffe, N.A.; Steele, D.; Witte, D.; Kirsten, M.; Louw, C.; Ngwira, B.; Victor, J.C.; Gillard, P.H.; Cheuvart, B.B.; et al. Effect of human rotavirus vaccine on severe diarrhea in African infants. *Malawi Med. J.* **2016**, *28*, 108–114, doi:10.1056/NEJMoa0904797.

73. Zaman, K.; Anh, D.D.; Victor, J.C.; Shin, S.; Yunus, M.; Dallas, M.J.; Podder, G.; Thiem, V.D.; Mai, L.T.P.; Luby, S.P.; et al. Efficacy of pentavalent rotavirus vaccine against severe rotavirus gastroenteritis in infants in developing countries in Asia: A randomised, double-blind, placebo-controlled trial. *Lancet* **2010**, *376*, 615–623, doi:10.1016/S0140-6736(10)60755-6.

74. Niendorf, S.; Faber, M.; Tröger, A.; Hackler, J.; Jacobsen, S. Diversity of noroviruses throughout outbreaks in Germany 2018. *Viruses* **2020**, *12*, 1–13, doi:10.3390/v12101157.

75. Carmona-Vicente, N.; Vila-Vicent, S.; Allen, D.; Gozalbo-Rovira, R.; Iturriza-Gómara, M.; Buesa, J.; Rodríguez-Díaz, J. Characterization of a Novel Conformational GII.4 Norovirus Epitope: Implications for Norovirus-Host Interactions. *J. Virol.* **2016**, *90*, 7703–7714, doi:10.1128/jvi.01023-16.

76. Chhabra, P.; de Graaf, M.; Parra, G.I.; Chan, M.C.W.; Green, K.; Martella, V.; Wang, Q.; White, P.A.; Katayama, K.; Vennema, H.; et al. Erratum: Updated classification of norovirus genogroups and genotypes (Journal of General Virology (2020) 100 (1393-1406) doi: 10.1099/jgv.0.001318). *J. Gen. Virol.* **2020**, *101*, 893, doi:10.1099/jgv.0.001475.

77. Tohma, K.; Lepore, C.J.; Gao, Y.; Ford-Siltz, L.A.; Parra, G.I. Population Genomics of GII.4 Noroviruses Reveal Complex Diversification and New Antigenic Sites Involved in the Emergence of Pandemic Strains. *bioRxiv* **2019**, 1–18, doi:10.1101/668772.

78. Kirkwood, C.D.; Bishop, R.F. Molecular detection of human calicivirus in young children hospitalized with acute gastroenteritis in Melbourne, Australia, during 1999. *J. Clin. Microbiol.* **2001**, *39*, 2722–2724, doi:10.1128/JCM.39.7.2722-2724.2001.

79. Morillo, S.G.; Timenetsky, M. do C.S.T.T. Norovirus: An overview. *Rev. Assoc. Med. Bras.* **2011**, *57*, 453–458, doi:10.1016/S0104-4230(11)70094-X.

80. da Silva Poló, T.; Peiró, J.R.; Mendes, L.C.N.; Ludwig, L.F.; de Oliveira-Filho, E.F.; Bucardo, F.; Huynen, P.; Melin, P.; Thiry, E.; Mauroy, A. Human norovirus infection in Latin America. *J. Clin. Virol.* **2016**, *78*, 111–119, doi:10.1016/j.jcv.2016.03.016.

81. Bartsch, S.M.; Lopman, B.A.; Ozawa, S.; Hall, A.J.; Lee, B.Y. Global economic burden of norovirus gastroenteritis. *PLoS One* **2016**, *11*, 1–16, doi:10.1371/journal.pone.0151219.

82. Cortes-Penfield, N.W.; Ramani, S.; Estes, M.K.; Atmar, R.L. Prospects and Challenges in the Development of a Norovirus Vaccine. *Clin. Ther.* **2017**, *39*, 1537–1549, doi:10.1016/j.clinthera.2017.07.002.

83. Bosch, A.; Pintó, R.M.; Guix, S. Human astroviruses. *Clin. Microbiol. Rev.* **2014**, *27*, 1048–1074, doi:10.1128/CMR.00013-14.

84. Kolawole, A.O.; Mirabelli, C.; Hill, D.R.; Svoboda, S.A.; Janowski, A.B.; Passalacqua, K.D.; Rodriguez, B.N.; Dame, M.K.; Freiden, P.; Berger, R.P.; et al. Astrovirus replication in human intestinal enteroids reveals multi-cellular tropism and an intricate host innate immune landscape. *PLoS Pathog.* **2019**, *15*, 1–30, doi:10.1371/journal.ppat.1008057.

85. De Benedictis, P.; Schultz-Cherry, S.; Burnham, A.; Cattoli, G. Astrovirus infections in humans and animals - Molecular biology, genetic diversity, and interspecies transmissions. *Infect. Genet. Evol.* **2011**, *11*, 1529–1544, doi:10.1016/j.meegid.2011.07.024.

86. Maldonado, Y.; Cantwell, M.; Old, M.; Hill, D.; De La Sanchez, M.L.; Logan, L.; Millan-Velasco, F.; Valdespino, J.L.; Sepulveda, J.; Matsui, S. Population-based prevalence of symptomatic and asymptomatic astrovirus infection in rural mayan infants. *J. Infect. Dis.* **1998**, *178*, 334–349, doi:10.1086/515625.

87. Lee, R.M.; Lessler, J.; Lee, R.A.; Rudolph, K.E.; Reich, N.G.; Perl, T.M.; Cummings, D.A.T. Incubation periods of viral gastroenteritis: A systematic review. *BMC Infect. Dis.* **2013**, *13*, doi:10.1186/1471-2334-13-446.

88. Oka, T.; Wang, Q.; Katayama, K.; Saifb, L.J. Comprehensive review of human sapoviruses. *Clin. Microbiol. Rev.* **2015**, *28*, 32–53, doi:10.1128/CMR.00011-14.

89. Lauritsen, K.T.; Hansen, M.S.; Johnsen, C.K.; Jungersen, G.; Böttiger, B. Repeated examination of natural sapovirus infections in pig litters raised under experimental conditions. *Acta Vet. Scand.* **2015**, *57*, 1–5,

doi:10.1186/s13028-015-0146-7.

90. Hansman, G.S.; Oka, T.; Sakon, N.; Takeda, N. Antigenic diversity of human sapoviruses. *Emerg. Infect. Dis.* **2007**, *13*, 1519–1525, doi:10.3201/eid1310.070402.

91. Makhaola, K.; Moyo, S.; Kebaabetswe, L.P. Distribution and genetic variability of sapoviruses in Africa. *Viruses* **2020**, *12*, 1–19, doi:10.3390/v12050490.

92. Mann, P.; Pietsch, C.; Liebert, U.G. Genetic diversity of sapoviruses among inpatients in germany, 2008−2018. *Viruses* **2019**, *11*, 1–14, doi:10.3390/v11080726.

93. Tohma, K.; Kulka, M.; Coughlan, S.; Green, K.Y.; Parra, G.I. Genomic analyses of human sapoviruses detected over a 40-year period reveal disparate patterns of evolution among genotypes and genome regions. *Viruses* **2020**, *12*, 5–13, doi:10.3390/v12050516.

94. Yamashita, T.; Sakae, K.; Tsuzuki, H.; Suzuki, Y.; Ishikawa, N.; Takeda, N.; Miyamura, T.; Yamazaki, S. Complete Nucleotide Sequence and Genetic Organization of Aichi Virus, a Distinct Member of the PicornaviridaeAssociated with Acute Gastroenteritis in Humans. *J. Virol.* **1998**, *72*, 8408–8412, doi:10.1128/jvi.72.10.8408-8412.1998.

95. Rivadulla, E.; Romalde, J.L. A Comprehensive Review on Human Aichi Virus. *Virol. Sin.* **2020**, *12250*, 501–516, doi:10.1007/s12250-020-00222-5.

96. Malik, Y.S.; Verma, A.K.; Kumar, N.; Touil, N.; Karthik, K.; Tiwari, R.; Bora, D.P.; Dhama, K.; Ghosh, S.; Hemida, M.G.; et al. Advances in Diagnostic Approaches for Viral Etiologies of Diarrhea: From the Lab to the Field. *Front. Microbiol.* **2019**, *10*, 1–18, doi:10.3389/fmicb.2019.01957.

97. Leland, D.S.; Ginocchio, C.C. Role of cell culture for virus detection in the age of technology. *Clin. Microbiol. Rev.* **2007**, *20*, 49–78, doi:10.1128/CMR.00002-06.

98. Johnston, S.P.; Ballard, M.M.; Beach, M.J.; Causer, L.; Wilkins, P.P. Evaluation of three commercial assays for detection of Giardia and Cryptosporidium organisms in fecal specimens. *J. Clin. Microbiol.* **2003**, *41*, 623–626, doi:10.1128/JCM.41.2.623-626.2003.

99. Beniac, D.R.; Siemens, C.G.; Wright, C.J.; Booth, T.F. A filtration based technique for simultaneous SEM and TEM sample preparation for the rapid detection of pathogens. *Viruses* **2014**, *6*, 3358–3371, doi:10.3390/v6093458.

100. Reller, L.B.; Weinstein, M.P. Special section: Medical microbiology. *Clin. Infect. Dis.* **2000**, *30*, 798, doi:10.1086/313790.

101. Church, D.L.; Miller, K.; Lichtenfeld, A.; Semeniuk, H.; Kirkham, B.; Laupland, K.; Elsayed, S. Screening for Giardia/Cryptosporidium infections using an enzyme immunoassay in a centralized regional microbiology

laboratory. *Arch. Pathol. Lab. Med.* **2005**, *129*, 754–759, doi:10.5858/2005-129-754-sfciua.

102. Liu, J.; Platts-Mills, J.A.; Juma, J.; Kabir, F.; Nkeze, J.; Okoi, C.; Operario, D.J.; Uddin, J.; Ahmed, S.; Alonso, P.L.; et al. Use of quantitative molecular diagnostic methods to identify causes of diarrhoea in children: a reanalysis of the GEMS case-control study. *Lancet* **2016**, *388*, 1291–1301, doi:10.1016/S0140-6736(16)31529-X.

103. Breurec, S.; Vanel, N.; Bata, P.; Chartier, L.; Farra, A.; Favennec, L.; Franck, T.; Giles-Vernick, T.; Gody, J.C.; Luong Nguyen, L.B.; et al. Etiology and Epidemiology of Diarrhea in Hospitalized Children from Low Income Country: A Matched Case-Control Study in Central African Republic. *PLoS Negl. Trop. Dis.* **2016**, *10*, 1–18, doi:10.1371/journal.pntd.0004283.

104. Presterl, E.; Zwick, R.H.; Reichmann, S.; Aichelburg, A.; Winkler, S.; Kremsner, P.G.; Graninger, W. Frequency and virulence properties of diarrheagenic Escherichia coli in children with diarrhea in Gabon. *Am. J. Trop. Med. Hyg.* **2003**, *69*, 406–410, doi:10.4269/ajtmh.2003.69.406.

105. Singh, P.; Metgud, S.C.; Roy, S.; Purwar, S. Evolution of diarrheagenic Escherichia coli pathotypes in India. *J. Lab. Physicians* **2019**, *11*, 346–351, doi:10.4103/jlp.jlp_58_19.

106. Ramya Raghavan, P.; Roy, S.; Thamizhmani, R.; Sugunan, A.P. Diarrheagenic Escherichia coli infections among the children of Andaman Islands with special reference to pathotype distribution and clinical profile. *J. Epidemiol. Glob. Health* **2017**, *7*, 305–308, doi:10.1016/j.jegh.2017.07.003.

107. Rasko, D. A., Webster, D. R., Sahl, J. W., Bashir, A., Boisen, N., Scheutz, F., … Waldor, M.K. Origins of the E. coli Strain Causing an Outbreak of Hemolytic–Uremic Syndrome in Germany. *New Engl. J. Med.* **2011**, doi:10.1056/NEJMoa1106920.

108. Gasparinho, C.; Mirante, M.C.; Centeno-Lima, S.; Istrate, C.; Mayer, A.C.; Tavira, L.; Nery, S.V.; Brito, M. Etiology of Diarrhea in Children Younger Than 5 Years Attending the Bengo General Hospital in Angola. *Pediatr. Infect. Dis. J.* **2016**, *35*, e28–e34, doi:10.1097/INF.0000000000000957.

109. Gatei, W.; Wamae, C.N.; Mbae, C.; Waruru, A.; Mulinge, E.; Waithera, T.; Gatika, S.M.; Kamwati, S.K.; Revathi, G.; Hart, C.A. Cryptosporidiosis: Prevalence, genotype analysis, and symptoms associated with infections in children in Kenya. *Am. J. Trop. Med. Hyg.* **2006**, *75*, 78–82, doi:10.4269/ajtmh.2006.75.78.

110. Mandomando, I.M.; Macete, E. V.; Ruiz, J.; Sanz, S.; Abacassamo, F.; Vallès, X.; Sacarlal, J.; Navia, M.M.; Vila, J.; Alonso, P.L.; et al. Etiology of diarrhea in children younger than 5 years of age admitted in a rural hospital of Southern Mozambique. *Am. J. Trop. Med. Hyg.* **2007**, *76*, 522–527, doi:10.4269/ajtmh.2007.76.522.

111. Duong, T. H., M. Kombila, D. Dufillot, D. Richard-Lenoble, M. M. Owono, M. Martz, D. Gendrel, E.E.& J.L.M. Role of cryptosporidiosis in infants in Gabon. Results of two pro-spective studies. *Bull. la Société Pathol. Exot.* **1991**, *84*, 635−644.

112. Lekana-Douki, S.E.; Kombila-Koumavor, C.; Nkoghe, D.; Drosten, C.; Drexler, J.F.; Leroy, E.M. Molecular epidemiology of enteric viruses and genotyping of rotavirus A, adenovirus and astrovirus among children under 5 years old in Gabon. *Int. J. Infect. Dis.* **2015**, *34*, 90–95, doi:10.1016/j.ijid.2015.03.009.

113. Ibrahim, S.B.; El-Bialy, A.A.; Mohammed, M.S.; El-Sheikh, A.O.; Elhewala, A.; Bahgat, S. Detection of Rotavirus in children with acute gastroenteritis in Zagazig University Hospitals in Egypt. *Electron. Physician* **2015**, *7*, 1227–1233, doi:10.14661/1227.

114. Leclerc, H.; Schwartzbrod, L.; Dei-Cas, E. Microbial agents associated with waterborne diseases. *Crit. Rev. Microbiol.* **2002**, *28*, 371–409, doi:10.1080/1040-840291046768.

115. Liu, J.; Platts-Mills, J.A.; Juma, J.; Kabir, F.; Nkeze, J.; Okoi, C.; Operario, D.J.; Uddin, J.; Ahmed, S.; Alonso, P.L.; et al. Use of quantitative molecular diagnostic methods to identify causes of diarrhoea in children: a reanalysis of the GEMS case-control study. *Lancet* **2016**, *388*, 1291–1301, doi:10.1016/S0140-6736(16)31529-X.

116. Koko, J.; Ambara, J.P.; Gahouma, D.; Ategbo, S. Epidemiology of acute bacterial diarrhea in children in Libreville, Gabon. *Arch. Pediatr.* **2013**, *20*, 432–433, doi:10.1016/j.arcped.2013.01.017.

117. Krumkamp, R.; Sarpong, N.; Schwarz, N.G.; Adelkofer, J.; Loag, W.; Eibach, D.; Hagen, R.M.; Adu-Sarkodie, Y.; Tannich, E.; May, J. Gastrointestinal Infections and Diarrheal Disease in Ghanaian Infants and Children: An Outpatient Case-Control Study. *PLoS Negl. Trop. Dis.* **2015**, *9*, 1–14, doi:10.1371/journal.pntd.0003568.

118. Harb, A.; O'Dea, M.; Abraham, S.; Habib, I. Childhood diarrhoea in the eastern mediterranean region with special emphasis on non-typhoidal salmonella at the human–food interface. *Pathogens* **2019**, *8*, doi:10.3390/pathogens8020060.

119. Chen, S.Y.; Tsai, C.N.; Chao, H.C.; Lai, M.W.; Lin, T.Y.; Ko, T.Y.; Chiu, C.H. Acute gastroenteritis caused by multiple enteric pathogens in children. *Epidemiol. Infect.* **2009**, *137*, 932–935, doi:10.1017/S095026880800160X.

120. Pijnacker, R.; van Pelt, W.; Vennema, H.; Kortbeek, L.M.; Notermans, D.W.; Franz, E.; Mughini-Gras, L. Clinical relevance of enteropathogen co-infections in preschool children—a population-based repeated cross-sectional study. *Clin. Microbiol. Infect.* **2019**, *25*, 1039.e7-1039.e13, doi:10.1016/j.cmi.2018.11.029.

121. Feghaly, R.E.E.; Stauber, J.L.; Tarr, P.I.; Haslam, D.B. Viral co-infections

are common and are associated with higher bacterial burden in children with clostridium difficile infection. *J. Pediatr. Gastroenterol. Nutr.* **2013**, *57*, 813–816, doi:10.1097/MPG.0b013e3182a3202f.

122. Kim, A.; Chang, J.Y.; Shin, S.; Yi, H.; Moon, J.S.; Ko, J.S.; Oh, S. Epidemiology and factors related to clinical severity of acute gastroenteritis in hospitalized children after the introduction of rotavirus vaccination. *J. Korean Med. Sci.* **2017**, *32*, 465–474, doi:10.3346/jkms.2017.32.3.465.

123. Agnamey, P.; Sarfati, C.; Pinel, C.; Rabodoniriina, M.; Kapel, N.; Dutoit, E.; Garnaud, C.; Diouf, M.; Garin, J.F.; Totet, A.; et al. Evaluation of four commercial rapid immunochromatographic assays for detection of Cryptosporidium antigens in stool samples: A blind multicenter trial. *J. Clin. Microbiol.* **2011**, *49*, 1605–1607, doi:10.1128/JCM.02074-10.

124. Van den Bossche, D.; Cnops, L.; Verschueren, J.; Van Esbroeck, M. Comparison of four rapid diagnostic tests, ELISA, microscopy and PCR for the detection of Giardia lamblia, Cryptosporidium spp. and Entamoeba histolytica in feces. *J. Microbiol. Methods* **2015**, *110*, 78–84, doi:10.1016/j.mimet.2015.01.016.

125. Bitilinyu-Bangoh, J.; Voskuijl, W.; Thitiri, J.; Menting, S.; Verhaar, N.; Mwalekwa, L.; De Jong, D.B.; Van Loenen, M.; Mens, P.F.; Berkley, J.A.; et al. Performance of three rapid diagnostic tests for the detection of Cryptosporidium spp. And Giardia duodenalis in children with severe acute malnutrition and diarrhoea. *Infect. Dis. Poverty* **2019**, *8*, 1–8, doi:10.1186/s40249-019-0609-6.

126. Rossi, G.; De Smet, M.; Khim, N.; Kindermans, J.M.; Menard, D. Performance of Rapid Diagnostic Testing in Patients with Suspected Malaria in Cambodia, a Low-Endemicity Country Aiming for Malaria Elimination. *Clin. Infect. Dis.* **2017**, *65*, 1769–1770, doi:10.1093/cid/cix625.

127. Uppal, B.; Singh, O.; Chadha, S.; Jha, A.K. A comparison of nested PCR assay with conventional techniques for diagnosis of intestinal cryptosporidiosis in AIDS cases from Northern India. *J. Parasitol. Res.* **2014**, *2014*, doi:10.1155/2014/706105.

128. Weitzel, T.; Dittrich, S.; Möhl, I.; Adusu, E.; Jelinek, T. Evaluation of seven commercial antigen detection tests for Giardia and Cryptosporidium in stool samples. *Clin. Microbiol. Infect.* **2006**, *12*, 656–659, doi:10.1111/j.1469-0691.2006.01457.x.

129. Helmy, Y.A.; Krücken, J.; Nöckler, K.; Von Samson-Himmelstjerna, G.; Zessin, K.H. Comparison between two commercially available serological tests and polymerase chain reaction in the diagnosis of Cryptosporidium in animals and diarrhoeic children. *Parasitol. Res.* **2014**, *113*, 211–216, doi:10.1007/s00436-013-3645-3.

130. Danišová, O.; Halánová, M.; Valenčáková, A.; Luptáková, L. Sensitivity, specificity and comparison of three commercially available immunological tests in the diagnosis of Cryptosporidium species in animals. *Brazilian J.*

Microbiol. **2018**, *49*, 177–183, doi:10.1016/j.bjm.2017.03.016.

131. Odimayo, M.S.; Olanrewaju, W.I.; Omilabu, S.A.; Adegboro, B. Prevalence of rotavirus-induced diarrhoea among children under 5 years in Ilorin, Nigeria. *J. Trop. Pediatr.* **2008**, *54*, 343–346, doi:10.1093/tropej/fmn081.

132. Iturriza-Gómara, M.; Jere, K.C.; Hungerford, D.; Bar-Zeev, N.; Shioda, K.; Kanjerwa, O.; Houpt, E.R.; Operario, D.J.; Wachepa, R.; Pollock, L.; et al. Etiology of Diarrhea among Hospitalized Children in Blantyre, Malawi, following Rotavirus Vaccine Introduction: A Case-Control Study. *J. Infect. Dis.* **2019**, *220*, 213–218, doi:10.1093/infdis/jiz084.

133. Duan, Z.J.; Liu, N.; Yang, S.H.; Zhang, J.; Sun, L.W.; Tang, J.Y.; Jin, Y.; Du, Z.Q.; Xu, J.; Wu, Q. Bin; et al. Hospital-based surveillance of rotavirus diarrhea in the peoples Republic of China, august 2003-july 2007. *J. Infect. Dis.* **2009**, *200*, doi:10.1086/605039.

134. Fidhow, A.M.; Samwel, A.; Ng'ang'a, Z.; Oundo, J.; Nyangao, J.; Wences, A. Molecular epidemiology and associated risk factors of rotavirus infection among children < 5 yrs hospitalized for acute gastroenteritis in North Eastern, Kenya, 2012. *Pan Afr. Med. J.* **2017**, *28*, 3, doi:10.11604/PAMJ.SUPP.2017.28.1.2486.

135. Gasparinho, C.; Piedade, J.; Mirante, M.C.; Mendes, C.; Mayer, C.; Nery, S.V.; Brito, M.; Istrate, C. Characterization of rotavirus infection in children with acute gastroenteritis in Bengo province, Northwestern Angola, prior to vaccine introduction. *PLoS One* **2017**, *12*, 1–19, doi:10.1371/journal.pone.0176046.

136. Nhampossa, T.; Mandomando, I.; Acacio, S.; Quintó, L.; Vubil, D.; Ruiz, J.; Nhalungo, D.; Sacoor, C.; Nhabanga, A.; Nhacolo, A.; et al. Diarrheal disease in rural Mozambique: Burden, risk factors and etiology of diarrheal disease among children aged 0-59 months seeking care at health facilities. *PLoS One* **2015**, *10*, 12–23, doi:10.1371/journal.pone.0119824.

137. Ndze, V.N.; Papp, H.; Achidi, E.A.; Gonsu, K.H.; László, B.; Farkas, S.; Kisfali, P.; Melegh, B.; Esona, M.D.; Bowen, M.D.; et al. One year survey of human rotavirus strains suggests the emergence of genotype G12 in Cameroon. *J. Med. Virol.* **2013**, *85*, 1485–1490, doi:10.1002/jmv.23603.

138. Mayindou, G.; Ngokana, B.; Sidibé, A.; Moundélé, V.; Koukouikila-Koussounda, F.; Christevy Vouvoungui, J.; Kwedi Nolna, S.; Velavan, T.P.; Ntoumi, F. Molecular epidemiology and surveillance of circulating rotavirus and adenovirus in Congolese children with gastroenteritis. *J. Med. Virol.* **2016**, *88*, 596–605, doi:10.1002/jmv.24382.

139. Tcheremenskaia, O.; Marucci, G.; De Petris, S.; Ruggeri, F.M.; Dovecar, D.; Sternak, S.L.; Matyasova, I.; Dhimolea, M.K.; Mladenova, Z.; Fiore, L.; et al. Molecular epidemiology of rotavirus in Central and Southeastern Europe. *J. Clin. Microbiol.* **2007**, *45*, 2197–2204, doi:10.1128/JCM.00484-07.

140. Moure, U.A.E.; Banga-Mingo, V.; Gody, J.C.; Mwenda, J.M.; Fandema, J.; Waku-Kouomou, D.; Manengu, C.; Koyazegbe, T.D.A.; Esona, M.D.; Bowen, M.D.; et al. Emergence of G12 and G9 rotavirus genotypes in the Central African Republic, January 2014 to February 2016. *BMC Res. Notes* **2018**, *11*, 5, doi:10.1186/s13104-017-3122-7.

141. Luchs, A.; Timenetsky, M. do C.S.T. Group A rotavirus gastroenteritis: post-vaccine era, genotypes and zoonotic transmission. *Einstein (Sao Paulo).* **2016**, *14*, 278–287, doi:10.1590/S1679-45082016RB3582.

142. Wylie, K.M.; Weinstock, G.M.; Storch, G.A. Emergence of rotavirus G12p[8] in St. Louis during the 2012-2013 rotavirus season. *J. Pediatric Infect. Dis. Soc.* **2015**, *4*, e84–e89, doi:10.1093/jpids/piu090.

143. Lestari, F.B.; Vongpunsawad, S.; Wanlapakorn, N.; Poovorawan, Y. Rotavirus infection in children in Southeast Asia 2008-2018: Disease burden, genotype distribution, seasonality, and vaccination. *J. Biomed. Sci.* **2020**, *27*, 1–19, doi:10.1186/s12929-020-00649-8.

144. Mwenda, J.M.; Ntoto, K.M.; Abebe, A.; Enweronu-Laryea, C.; Amina, I.; Mchomvu, J.; Kisakye, A.; Mpabalwani, E.M.; Pazvakavambwa, I.; Armah, G.E.; et al. Burden and epidemiology of rotavirus diarrhea in selected african countries: Preliminary results from the african rotavirus surveillance network. *J. Infect. Dis.* **2010**, *202*, 5–11, doi:10.1086/653557.

145. Motamedi-Rad, M.; Farahmand, M.; Arashkia, A.; Jalilvand, S.; Shoja, Z. VP7 and VP4 genotypes of rotaviruses cocirculating in Iran, 2015 to 2017: Comparison with cogent sequences of Rotarix and RotaTeq vaccine strains before their use for universal mass vaccination. *J. Med. Virol.* **2020**, *92*, 1110–1123, doi:10.1002/jmv.25642.

146. Mouna, B.H.F.; BenHamida-Rebaï, M.; Heylen, E.; Zeller, M.; Moussa, A.; Kacem, S.; Van Ranst, M.; Matthijnssens, J.; Trabelsi, A. Sequence and phylogenetic analyses of human rotavirus strains: Comparison of VP7 and VP8* antigenic epitopes between Tunisian and vaccine strains before national rotavirus vaccine introduction. *Infect. Genet. Evol.* **2013**, *18*, 132–144, doi:10.1016/j.meegid.2013.05.008.

147. Morozova, O. V.; Sashina, T.A.; Fomina, S.G.; Novikova, N.A. Comparative characteristics of the VP7 and VP4 antigenic epitopes of the rotaviruses circulating in Russia (Nizhny Novgorod) and the Rotarix and RotaTeq vaccines. *Arch. Virol.* **2015**, *160*, 1693–1703, doi:10.1007/s00705-015-2439-6.

148. Ouédraogo, N.; Kaplon, J.; Bonkoungou, I.J.O.; Traoré, A.S.; Pothier, P.; Barro, N.; Ambert-Balay, K. Prevalence and genetic diversity of enteric viruses in children with diarrhea in Ouagadougou, Burkina Faso. *PLoS One* **2016**, *11*, 1–22, doi:10.1371/journal.pone.0153652.

149. Zhang, S.X.; Zhou, Y.M.; Xu, W.; Tian, L.G.; Chen, J.X.; Chen, S.H.; Dang, Z.S.; Gu, W.P.; Yin, J.W.; Serrano, E.; et al. Impact of co-infections with enteric pathogens on children suffering from acute diarrhea in southwest

China. *Infect. Dis. Poverty* **2016**, *5*, 1–13, doi:10.1186/s40249-016-0157-2.

150. Aksara Thongprachum , Sayaka Takanashi, Angela F C Kalesaran, Shoko Okitsu, Masashi Mizuguchi, Satoshi Hayakawa, H.U. Four-year study of viruses that cause diarrhea in Japanese pediatric outpatients. *J Med Virol* **2015**, doi:10.1002/jmv.

151. Alcalá, A.C.; Pérez, K.; Blanco, R.; González, R.; Ludert, J.E.; Liprandi, F.; Vizzi, E. Molecular detection of human enteric viruses circulating among children with acute gastroenteritis in Valencia, Venezuela, before rotavirus vaccine implementation. *Gut Pathog.* **2018**, *10*, 1–12, doi:10.1186/s13099-018-0232-2.

152. El Qazoui, M.; Oumzil, H.; Baassi, L.; El Omari, N.; Sadki, K.; Amzazi, S.; Benhafid, M.; El Aouad, R. Rotavirus and norovirus infections among acute gastroenteritis children in Morocco. *BMC Infect. Dis.* **2014**, *14*, 1–9, doi:10.1186/1471-2334-14-300.

153. Ayukekbong, J.; Lindh, M.; Nenonen, N.; Tah, F.; Nkuo-Akenji, T.; Bergström, T. Enteric viruses in healthy children in Cameroon: Viral load and genotyping of norovirus strains. *J. Med. Virol.* **2011**, *83*, 2135–2142, doi:10.1002/jmv.22243.

154. Arowolo, K.O.; Ayolabi, C.I.; Lapinski, B.; Santos, J.S.; Raboni, S.M. Epidemiology of enteric viruses in children with gastroenteritis in Ogun State, Nigeria. *J. Med. Virol.* **2019**, *91*, 1022–1029, doi:10.1002/jmv.25399.

155. Taghinejad, M.; Ghaderi, M.; Mousavi-Nasab, S.D. Aichivirus with acute gastroenteritis in Iran. *Pediatr. Infect. Dis. J.* **2020**, *39*, 576–579, doi:10.1097/INF.0000000000002638.

156. Ahmed, S.M.; Hall, A.J.; Robinson, A.E.; Verhoef, L.; Premkumar, P.; Parashar, U.D.; Koopmans, M.; Lopman, B.A. Global prevalence of norovirus in cases of gastroenteritis: A systematic review and meta-analysis. *Lancet Infect. Dis.* **2014**, *14*, 725–730, doi:10.1016/S1473-3099(14)70767-4.

157. Shanker, S.; Choi, J.-M.; Sankaran, B.; Atmar, R.L.; Estes, M.K.; Prasad, B.V. V. Structural Analysis of Histo-Blood Group Antigen Binding Specificity in a Norovirus GII.4 Epidemic Variant: Implications for Epochal Evolution. *J. Virol.* **2011**, *85*, 8635–8645, doi:10.1128/jvi.00848-11.

158. Bruggink, L.D.; Dunbar, N.L.; Marshall, J.A. Emergence of GII.e as a major ORF 1 norovirus genotype and its associated ORF 2 GII.4 variant forms. *Infect. Genet. Evol.* **2014**, *22*, 157–163, doi:10.1016/j.meegid.2014.01.025.

159. Thanh, H.D.; Than, V.T.; Nguyen, T.H.; Lim, I.; Kim, W. Emergence of norovirus GII.17 variants among children with acute gastroenteritis in South Korea. *PLoS One* **2016**, *11*, doi:10.1371/journal.pone.0154284.

160. Bruggink, L.D.; Moselen, J.M.; Marshall, J.A. Genotype analysis of noroviruses associated with gastroenteritis outbreaks in childcare centres, Victoria, Australia, 2012-2015. *Epidemiol. Infect.* **2017**, *145*, 1933–1941,

doi:10.1017/S0950268817000681.

161. Mans, J.; Murray, T.Y.; Nadan, S.; Netshikweta, R.; Page, N.A.; Taylor, M.B. Norovirus diversity in children with gastroenteritis in South Africa from 2009 to 2013: GII.4 variants and recombinant strains predominate. *Epidemiol. Infect.* **2016**, *144*, 907–916, doi:10.1017/S0950268815002150.

162. Makhaola, K.; Moyo, S.; Lechiile, K.; Goldfarb, D.M.; Kebaabetswe, L.P. Genetic and epidemiological analysis of norovirus from children with gastroenteritis in Botswana, 2013-2015. *BMC Infect. Dis.* **2018**, *18*, 1–8, doi:10.1186/s12879-018-3157-y.

163. Wu, X.; Han, J.; Chen, L.; Xu, D.; Shen, Y.; Zha, Y.; Zhu, X.; Ji, L. Prevalence and genetic diversity of noroviruses in adults with acute gastroenteritis in Huzhou, China, 2013–2014. *Arch. Virol.* **2015**, *160*, 1705–1713, doi:10.1007/s00705-015-2440-0.

164. Chen, C.; Yan, J.B.; Wang, H.L.; Li, P.; Li, K.F.; Wu, B.; Zhang, H. Molecular epidemiology and spatiotemporal dynamics of norovirus associated with sporadic acute gastroenteritis during 2013–2017, Zhoushan Islands, China. *PLoS One* **2018**, *13*, 1–14, doi:10.1371/journal.pone.0200911.

165. Widdowson, M.A.; Sulka, A.; Bulens, S.N.; Beard, R.S.; Chaves, S.S.; Hammond, R.; Salehi, E.D.P.; Swanson, E.; Totaro, J.; Woron, R.; et al. Norovirus and foodborne disease, United States, 1991-2000. *Emerg. Infect. Dis.* **2005**, *11*, 95–102, doi:10.3201/eid1101.040426.

166. Japhet, M.O.; Famurewa, O.; Adesina, O.A.; Opaleye, O.O.; Wang, B.; Höhne, M.; Bock, C.T.; Mas Marques, A.; Niendorf, S. Viral gastroenteritis among children of 0-5 years in Nigeria: Characterization of the first Nigerian aichivirus, recombinant noroviruses and detection of a zoonotic astrovirus. *J. Clin. Virol.* **2019**, *111*, 4–11, doi:10.1016/j.jcv.2018.12.004.

167. Pang, X.L.; Vesikari, T. Human astrovirus-associated gastroenteritis in children under 2 years of age followed prospectively during a rotavirus vaccine trial. *Acta Paediatr. Int. J. Paediatr.* **1999**, *88*, 532–536, doi:10.1080/08035259950169549.

168. Jeong, A.Y.; Jeong, H.S.; Jo, M.Y.; Jung, S.Y.; Lee, M.S.; Lee, J.S.; Jee, Y.M.; Kim, J.H.; Cheon, D.S. Molecular epidemiology and genetic diversity of human astrovirus in South Korea from 2002 to 2007. *Clin. Microbiol. Infect.* **2011**, *17*, 404–408, doi:10.1111/j.1469-0691.2010.03263.x.

169. Shrestha, S.K.; Shrestha, J.; Andreassen, A.K.; Strand, T.A.; Dudman, S.; Dembinski, J.L. Genetic Diversity of Astrovirus in Children From a Birth Cohort in Nepal. *Front. Microbiol.* **2021**, *11*, 1–9, doi:10.3389/fmicb.2020.588707.

170. Lekana-Douki, S.E.; Kombila-Koumavor, C.; Nkoghe, D.; Drosten, C.; Drexler, J.F.; Leroy, E.M. Molecular epidemiology of enteric viruses and genotyping of rotavirus A, adenovirus and astrovirus among children under

5 years old in Gabon. *Int. J. Infect. Dis.* **2015**, *34*, 90–95, doi:10.1016/j.ijid.2015.03.009.

171. Cilli, A.; Luchs, A.; Leal, E.; Gill, D.; Milagres, F.A.D.P.; Komninakis, S.V.; Brustulin, R.; Rodrigues Teles, M.D.A.; Sayão Lobato, M.C.A.B.; Das Chagas, R.T.; et al. Human sapovirus GI.2 and GI.3 from children with acute gastroenteritis in northern Brazil. *Mem. Inst. Oswaldo Cruz* **2019**, *114*, 10–13, doi:10.1590/0074-02760180574.

172. Ambert-Balay, K.; Lorrot, M.; Bon, F.; Giraudon, H.; Kaplon, J.; Wolfer, M.; Lebon, P.; Gendrel, D.; Pothier, P. Prevalence and genetic diversity of Aichi virus strains in stool samples from community and hospitalized patients. *J. Clin. Microbiol.* **2008**, *46*, 1252–1258, doi:10.1128/JCM.02140-07.

173. Nielsen, A.C.Y.; Gyhrs, M.L.; Nielsen, L.P.; Pedersen, C.; Böttiger, B. Gastroenteritis and the novel picornaviruses aichi virus, cosavirus, saffold virus, and salivirus in young children. *J. Clin. Virol.* **2013**, *57*, 239–242, doi:10.1016/j.jcv.2013.03.015.

174. Kaikkonen, S.; Räsänen, S.; Rämet, M.; Vesikari, T. Aichi virus infection in children with acute gastroenteritis in Finland. *Epidemiol. Infect.* **2010**, *138*, 1166–1171, doi:10.1017/S0950268809991300.

175. Rivadulla, E.; Romalde, J.L. Correction to: A Comprehensive Review on Human Aichi Virus. *Virol. Sin.* **2020**, *12250*, 15782, doi:10.1007/s12250-020-00324-0.

7. DECLARATION OF CONTRIBUTIONS

The doctoral dissertation entitled “Diagnosis and genetic characteristics of potential pathogens in children under five years of age with diarrhea” is based on four publications (Publication Nr.1: The American Journal of Tropical Medicine and Hygiene. 2021 Jul 7;105(1):254-260. PMID: 34232911; Publication Nr.2: PLoS Neglected Tropical Diseases. 2020 Jul 13;14(7): e0008448. PMID: 32658930; Publication Nr.3: Viruses. 2021 Mar 24;13(4):545. PMID: 33805214 and Publication Nr.4: EBioMedicine. 2021 Oct 24; 73:103648. PMID: 34706308) accomplished by Mr. Gédéon Prince Manouana as a first author, which form the backbone of this dissertation. We declare that Mr. Gédéon Prince Manouana contributed substantially to all four manuscripts as follows.

Publication 1

Prevalence of Pathogens in Young Children Presenting to Hospital with Diarrhea from Lambaréné, Gabon.

- Conceived and designed the study
- Participated in the recruitment of patients and data collection
- Performed the analysis of data
- Drafted, and improved the manuscript for publication

Publication 2

Performance of a rapid diagnostic test for the detection of Cryptosporidium spp. in African children admitted to hospital with diarrhea.

- Contributed to the study design
- Participated in the recruitment of patients
- Performed RDT and PCR, data analysis, and interpretation
- Prepared manuscript

Publication 3

Genetic Diversity of Enteric Viruses in Children under Five Years Old in Gabon

- Conceived and designed the study
- Participated in the recruitment of patients and data collection

• Performed PCR, sequencing, data analysis, and interpretation

• Drafted, and improved the manuscript for publication

Publication 4

Molecular surveillance and genetic divergence of rotavirus A antigenic epitopes in Gabonese children with acute gastroenteritis.

• Conceived and designed the study

• Participated in the recruitment of patients and data collection

• Performed PCR, sequencing, data analysis, and interpretation

• Prepared manuscript

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