

# Viral metagenomics analysis of stool specimens from children with unresolved gastroenteritis in Qatar

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## ABSTRACT

Acute gastroenteritis (AGE) is associated with significant global morbidity and mortality, especially among children under five years of age. Viruses are well established as etiologic agents of gastroenteritis since they are the most common pathogens that contribute to the disease burden in developing countries. Despite the advances in molecular diagnosis, a substantial proportion of AGE etiology remain unresolved. We implemented a viral metagenomics pipeline to determine the potential viral etiology associated with AGE among children under the age of five years in Qatar with undiagnosed etiology. Following enriching for the viral genome, ~1.3 billion sequences were generated from 89 stool specimens using the Illumina HiSeq platform, of which 7% were mapped to viral genomes. Human viruses were detected in 34 specimens (38.2%); 14 were adenovirus, nine coxsackievirus A16, five rotavirus (G9P[8] and G4P[8]), four norovirus (GI), one influenza A virus (H3), and one respiratory syncytial virus A (RSVA). In conclusion, the viral metagenomics approach is useful for determining AGE's etiology when routine molecular diagnostic assays fail.

## 1. Introduction

Acute gastroenteritis constitutes a significant health burden worldwide, especially in children residing in developing countries (Lakhan et al., 2013). (Chow et al., 2010). Diarrhea ranks as the fourth cause of mortality among children after pre-term birth complications, lower respiratory infections, and intrapartum diseases (Perin et al., 2022). Viruses including rotavirus, norovirus, astrovirus, and adenovirus are considered the major cause of diarrhea in children. (Clark and Kendrick, 2004; do Socorro Fôro Ramos et al., 2021; Fields et al., 2007; Middleton, 1996; Oude Munnink and van der Hoek, 2016). Worldwide, rotavirus infections remain a major cause of diarrhea mortality in children younger than five years (Troeger et al., 2018). While already lifesaving in developed countries, rotavirus vaccines are less effective in

developing countries (Tissera et al., 2017) due to higher early in life transmission rates of rotavirus infection (Steele et al., 2016) and inadequate vaccination coverage or accessibility (Rheingans et al., 2012). Additionally, bacteria such as *Campylobacter* spp. and *Clostridium difficile* as well as parasites such as *Giardia lamblia* and *Cryptosporidium* spp. are important causes of AGE (Meyer et al., 2020).

The Global Burden of Diseases (GBD) study assessed the deaths and etiologies of diarrhea in 195 countries between 1990 and 2016. The authors reported that the incidence of rotavirus infection was the highest in children younger than five years with diarrhea, followed by *campylobacter* spp., enterotoxigenic *E. coli* and shigella (Troeger et al., 2018). Similarly, the Global Enteric Multicenter Study (GEMS) reported that the majority of moderate-to-severe diarrhea among children under five years of age in sub-Saharan Africa and South Asia was attributable

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to four pathogens namely rotavirus, *Cryptosporidium* spp., enterotoxigenic *E. coli* producing heat-stable toxin, and *Shigella* spp. (Kotloff et al., 2019).

Despite the advances in molecular diagnosis, the etiology of many outbreaks remains unsolved using conventional diagnostic methods (Kotloff et al., 2013; Moore et al., 2015). These commercially available assays target known or common pathogens associated with the illness, which makes them unsuitable for the detection of variant strains and unusual or novel pathogens (Staheli et al., 2011). Next-generation sequencing (NGS) enables metagenomics-based identification of viruses by sequencing random fragments of all genomes present in a clinical or environmental sample. Metagenomics, defined as the sequence-based analysis of the whole collection of genomes directly isolated from a sample, enables the diagnosis of clinical specimens without a priori knowledge of the potential disease-causing pathogen(s) (Handelsman et al., 1998). Accordingly, viral metagenomics enables the identification of potentially any viral sequence in the clinical sample, including cultivable or uncultivable, known, unexpected, or novel viruses (Moore et al., 2015). In this study, we employed a virus-enrichment metagenomics approach to determine the potential etiologies of undiagnosed gastroenteritis cases using stool specimens obtained from children under five years of age.

## 2. Materials and methods

### 2.1. Ethical approval and sample collection

The study was approved by the Institutional Review Boards of the American University of Beirut and Hamad Medical Corporation. Written informed consent was obtained from the children's parents to use their samples in the study.

Stool samples were collected from children younger than five years of age who presented with diarrhea at the Pediatric Emergency Center (PEC) of Hamad Medical Corporation (HMC) in Qatar. The study was conducted between June 2016 and August 2019 and included 774 stool specimens from children under five with gastroenteritis (Fig. 1). All patients were residents of Qatar at the time of the study. Specimens were

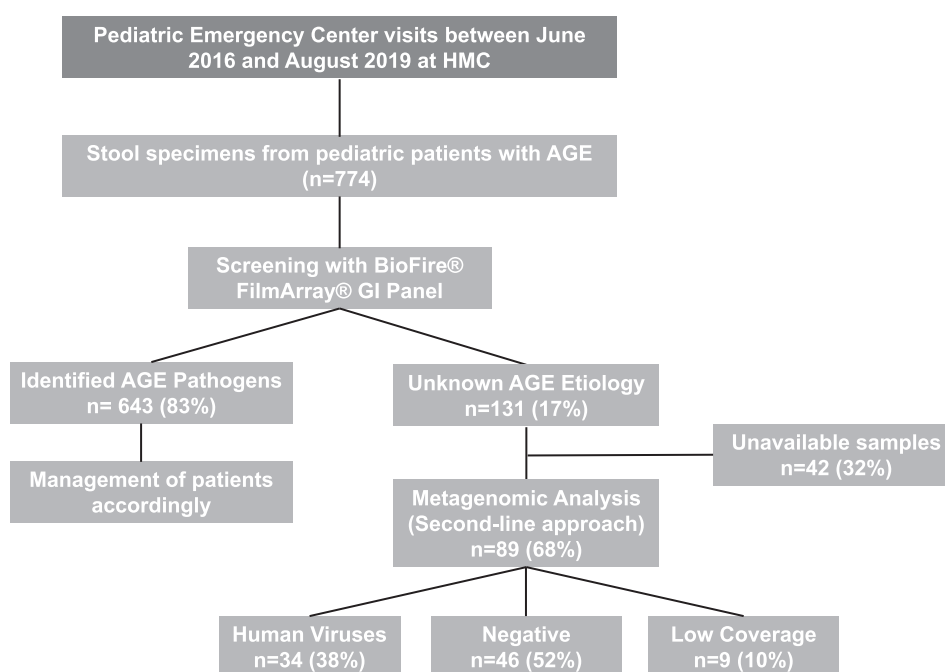
screened using BioFire® FilmArray® Gastrointestinal (GI) Panel,

(BIOFIRE®, Cambridge, USA), which detects 22 most common gastrointestinal pathogens, including five viruses (adenovirus F40/41, astrovirus, norovirus GI/GII, rotavirus A, sapovirus I, II, IV, and V), four parasites (*Cryptosporidium*, *Cyclospora cayetanensis*, *Entamoeba histolytica*, *Giardia lamblia*) and 13 bacteria (*Campylobacter* [jejuni, coli, and upsaliensis], *Clostridium difficile* toxin A/B; *Plesiomonas shigelloides*, *Salmonella*, *Yersinia enterocolitica*, enteroaggregative *E. coli*, enteropathogenic *E. coli*, enterotoxigenic *E. coli* lt/st, Shiga-like toxin-producing *E. coli* stx1/stx2, *E. coli* O157, *Shigella*/enteroinvasive *E. coli*, *Vibrio* [parahaemolyticus, vulnificus, and cholerae], *Vibrio cholerae*). In total, 131 out of the 774 specimens (17%) were of unknown etiology (negative by the panel); 89 of these specimens were available for metagenomics analysis. In addition, three norovirus and two rotavirus-confirmed specimens were included in the study to validate the protocol. The specimens were frozen and shipped on ice from Qatar University to the laboratory at the American University of Beirut and stored at -80 °C until further analysis.

Demographic and clinical data were extracted from the patients' medical records. All gastroenteritis symptoms were integrated into a composite disease severity scale on 20 points termed the Vesikari score (Ruuska and Vesikari, 1990). The score contains seven variables: maximum number of stools per day, diarrhea duration, maximum number of vomiting per day, vomiting duration, temperature, dehydration, and treatment.

### 2.2. Viral genome enrichment and amplification

An aliquot (40 g) of the stool sample was washed in 0.89% NaCl buffer (10% w/v for solid and v/v for liquid stool suspensions) and centrifuged at 4000g to remove debris. The supernatant was collected and subjected to two additional centrifugation steps. The sample was then passed through a 0.22 µm filter to remove host and bacterial cells, and the filtrates were treated with RNase (Thermo Fisher Scientific, USA). Total RNA from the sample was extracted using the QIAamp Viral RNA Mini kit (Qiagen, Germany) and treated with TURBO DNase (Turbo DNA-Free Kit, Invitrogen, USA). The viral RNA and mRNA were then



**Fig. 1.** The flowchart of the study.

AGE, Acute gastroenteritis; HMC, Hamad Medical Corporation; GI, Gastrointestinal.

reverse-transcribed using the SuperScript IV first-strand synthesis system (Thermo Fisher Scientific, USA), and the cDNA was then converted to double-stranded (ds-DNA) by adding 5'-end exonuclease Klenow fragment (Promega) (3' → 5' exo) as per the kit instructions. The ds-DNA was purified by using the QIAquick purification kit (Qiagen, Germany). Purified DNA was amplified to further enrich the viral genome, using the REPLI-g Single Cell Kit (Qiagen, Germany), which allows for primer independent amplification. The libraries were prepared according to the Nextera XT protocol (Illumina) and sequenced on the Illumina HiSeq platform (Illumina).

### 2.3. Data collection, storage, processing, and analysis

A high-throughput analysis pipeline was set up on Galaxy hosted on a local server. The pipeline consisted of the following steps: quality check of the Fastq files using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) followed by trimming low-quality bases using Trimmomatic (Bolger et al., 2014). Trimmed sequences were aligned and mapped against reference sequences using Bowtie 2 (<https://sourceforge.net/projects/bowtie-bio/files/bowtie2/>). The sequences were first mapped to a reference human genome. Unaligned reads were then mapped to rRNA and to reference bacterial genomes to enrich for viral sequences. The unaligned reads from the previous step were then mapped to a locally hosted, indexed viral database downloaded from GenBank. Viral reads were assembled into contigs using IDBA (A Practical Iterative de Bruijn Graph De Novo Assembler) (Peng et al., 2010). Contigs of a minimum length of 100 bp were aligned to a local GenBank viral database using MegaBLAST, and any hit with an e-value of  $\leq 1e-05$  (Ren et al., 2017) was considered a significant hit and further assessed. The genotypes of the identified viruses were assigned using the closest MegaBLAST hit corresponding to the longest contigs mapped to the genes used as the basis for genotyping.

### 3. Results

The demographic and clinical characteristics of the patients whose AGE etiology was identified by metagenomics are summarized in Table 1. The mean age of the patients was ~2 years and the majority (70%) were under 3-year-of-age (Table 1). Nearly two thirds (64%) of the children in the cohort had received the rotavirus vaccine. Around half were treated with analgesics (52%) and 22% received antibiotics. The average Vesikari score of the patients was 8.58. The majority of patients developed diarrhea (97%) and vomiting (76%). The average of the highest recorded body temperature was 37.35 °C, and most children experienced mild to moderate dehydration.

Five BioFire-positive specimens (three norovirus and two rotavirus-positive) were used to validate the metagenomics protocol and pipeline. We successfully confirmed the viral etiology in all of the five samples, which generated a total of 88,216,178 sequence reads, 15% of which mapped to the virus database (data not shown). The 89 BioFire-negative stool specimens generated approximately 1.3 billion sequence reads, with an average of 14.5 million reads per sample. Of these, 89.7 million (7%) were mapped to the virus database, and the remaining could not be assigned. The viral reads were assembled into contigs and aligned to a local GenBank viral database using MegaBLAST. We successfully associated a virus etiology to 34 out of the 89 (38%) AGE cases that were previously unresolved. Moreover, 46 samples were negative (52%) and nine (10%) had low coverage and could not be processed. The detected viruses belonged to the following families: *Adenoviridae*, *Caliciviridae*, *Orthomyxoviridae*, *Picornaviridae*, *Pneumoviridae*, and *Sedoreoviridae* (Fig. 2A, Table S1).

Adenovirus was the most detected virus accounting for 16% (14 of 89) of the undiagnosed cases, followed by coxsackievirus 10% (9 of 89), rotavirus 6% (5 of 89), and norovirus 4% (4 of 89) (Fig. 2B). Shedding of respiratory viruses was also detected, albeit less frequently, including one specimen each of influenza A (H3) virus and respiratory syncytial

**Table 1**

The demographic and clinical characteristics of patients with AGE associated with a viral etiology using metagenomics.

	Patients with a viral etiology*
<b>Demographic Information</b>	
Age	
<1 year	11/34 (32%)
1–3 years	14/34 (41%)
3–5 years	9/34 (27%)
Mean age in years, (SD)	1.93 (1.44)
Male sex	17/29 (59%)
<b>Symptoms</b>	
Diarrhea	33/34 (97%)
Vomiting	26/34 (76%)
Fever	16/34 (47%)
Mean highest recorded temperature in °C, (SD)	37.35 (0.706)
Mild to moderate Dehydration	31/33 (94%)
Blood in stool	1/34 (3%)
<b>AGE Severity</b>	
Vesikari Score 0–8 (mild)	15/34 (44%)
Vesikari Score 9–10 (moderate)	14/34 (41%)
Vesikari Score $\geq 11$ (severe)	5/34 (15%)
Average Vesikari Score (SD)	8.58 (2.96)
Rota vaccine	21/33 (64%)
<b>Treatment</b>	
Antibiotics	7/32 (22%)
Intravenous fluid	8/29 (28%)
Electrolyte replacement solution	4/29 (14%)
Antiemetic	7/29 (24%)
Analgesic painkiller	15/29 (52%)
Vitamin D	3/29 (10%)

Abbreviations: AGE, Acute Gastroenteritis; SD, Standard Deviation.

\* Data was missing for some specimens.

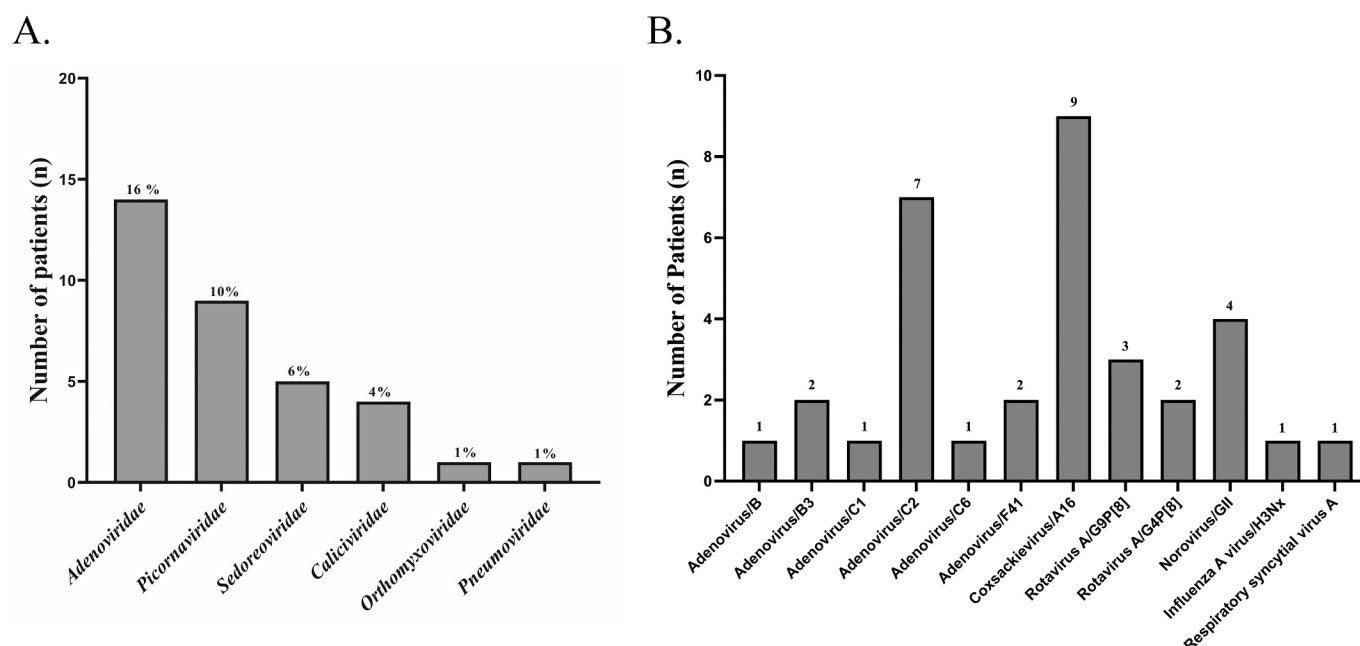
virus A (RSVA), a member of the *Human orthopneumovirus* species. Adenoviruses belonged to multiple genotypes including B3, F41, C1, C2, and C6 (Table S1). Noroviruses were of the GII genotype, rotaviruses were G9P[8] and G4P[8], and all detected coxsackieviruses were A16 (Table S1).

Notably, vaccine-related poliovirus sequences were co-detected in 17 out of the 34 the metagenomics-positive stool specimens. Poliovirus detection was the highest in the 0–18 months age group (11/17, 65%; all positives were 5 months and above) followed by the 18–47 months (4/11, 36%) and 48–60 months (2/6, 33%) age groups.

Plant viruses were detected in several stool specimens including the cucumber green mottle virus, tomato mosaic virus, pepper mild mottle virus, cucurbit chlorotic yellows virus, and pituitosporium cryptic virus-1 (data not shown). However, we did not consider these viruses as potential etiologies due to their undefined role as human pathogens.

### 4. Discussion

Previous studies have shown that an etiology cannot be determined for almost one-third of AGE cases (Farfán-García et al., 2020; Wikswo et al., 2015). A recent study in Qatar identified a viral or bacterial etiology in only 57.2% of acute gastroenteritis cases using a multiplex PCR assay, whereas the remaining cases could not be diagnosed (Al-Thani et al., 2013). In this cohort, an etiology could not be associated with 17% of the specimens using a superplex PCR panel. The failure to detect common AGE viruses by PCR-based molecular techniques, including the BioFire® FilmArray® GI Panel used in our study, can be attributed to their targeted and primer-based nature (Kotloff et al., 2019, 2013; Moore et al., 2015). Here, we used viral metagenomics to resolve the potential viral etiology in undiagnosed AGE cases. We successfully identified a viral pathogen in nearly one-third of these cases (38%), most of which were viruses commonly implicated in diarrhea. In this study,



**Fig. 2.** The incidence and genotypic distribution of the different viral etiologies identified in patients with unresolved AGE using metagenomics analysis. (A) Breakdown of AGE etiologies identified in patients with unresolved AGE by virus family ( $n = 89$ ). (B) Frequency of detected viruses ( $n = 34$ ).

the viral reads proportion (7%) was similar to that obtained from stool samples (5%) in a study from Kuwait (Mohammad et al., 2020). Other studies obtained higher proportions of reads derived from viruses (Victoria et al., 2009; Yang et al., 2022). However, they did not exclude from metagenomic evaluation, the samples that were confirmed positive by conventional testing. Such samples may have contributed to the increased percentage of viral reads. Similarly, our PCR-positive specimens yielded twice as many viral reads as compared to the negative ones. In another study by Fernandez-Cassi et al., the range of viral reads was between 29% and 50% for children aged less than one year (Fernandez-Cassi et al., 2020). Nonetheless, the authors pooled several samples together before metagenomic testing. Moreover, they purified their extracted DNA using a Zymo DNA clean and concentrator, which may have improved the efficiency of the downstream sequencing application.

Adenovirus accounted for the majority of AGE cases in our study. These belonged to subgroups B, C, and F known to be associated with gastrointestinal infections (Ghebremedhin, 2014). Al-Thani et al. previously showed that adenovirus is the third most common cause of acute gastroenteritis in Qatar, particularly among children (Al-Thani et al., 2013). Coxsackievirus type A16 was the second most common virus in stool specimens accounting for 10.1% of the cases in our study. Coxsackievirus A16 is the most common cause of the hand, foot, and mouth disease (HFMD), which can manifest as a respiratory infection or gastroenteritis (Gonzalez et al., 2019; Mao et al., 2014; Zhou et al., 2016). Unfortunately, we do not have data on non-gastroenteric symptoms to determine whether patients in our study with coxsackieviruses presented with HFMD-associated mouth ulcers and skin rash. Enteroviruses (including coxsackievirus A16) are often overlooked and not included in GI diagnostic assays or multiplex panels, including the one used in our study resulting in underestimating their role in acute gastroenteritis (Binnicker, 2015; Moore et al., 2015; Panchalingam et al., 2012; Zhou et al., 2016).

A subset of unresolved AGE cases in our study was attributed to norovirus and rotavirus, previously shown to account for the majority of diarrheal cases in Qatar (Al-Thani et al., 2013). The norovirus specimens were of the GII genotype, which is predominant in the Middle East and North Africa (MENA), including Qatar, where it accounted for 99.8% of all norovirus infections (Kreidieh et al., 2017; Mathew et al., 2019).

Regarding rotavirus, we recently reported that G3P[8] accounted for one-third of rotavirus-associated AGE among children under five in Qatar (Mathew et al., 2021). Here, the rotavirus specimens were of the G9P[8] or G4P[8] genotypes, previously reported to account for 8.7% and 11.6%, respectively of rotavirus-associated AGE among children in Qatar (Mathew et al., 2021). These two genotypes are also among the most common genotypes reported in the Middle East and North Africa (MENA) region (Ali et al., 2016).

Influenza A virus and RSV were detected in two separate specimens. Gastrointestinal symptoms including diarrhea, vomiting, and abdominal pain are frequently reported among influenza-confirmed patients (Assaf-Casals et al., 2020). An incidence of diarrhea as high as 56% has been reported among influenza patients (Minodier et al., 2019). Al khatib et al. found influenza RNA in 41% of stool specimens from influenza-positive patients, and 6% shed viable viruses (Al Khatib et al., 2021). A systematic review reported that the pooled incidence of influenza shedding in stools was 20.6% (Minodier et al., 2015). On the other hand, we previously showed that RSV is associated with diarrhea in pediatric cancer patients (Soudani et al., 2019). Shedding of RSV in the stool has also been documented in other studies (von Linstow et al., 2004). These findings suggest a potential etiologic role for respiratory viruses in AGE especially when enteric viruses cannot be detected. The mechanism(s) by which respiratory viruses induce GI symptoms remain undetermined. One potential pathobiological mechanism is that influenza viruses may replicate inside intestinal cells leading to cellular dysfunction (Aleandri et al., 2015; Elbashir et al., 2020). The development of GI symptoms may also be attributed to alterations in the intestinal microbiota profile by influenza infection. Utilizing an influenza mouse model, Deriu et al. demonstrated that the infection triggers a “dysbiotic” microenvironment in the gut while enriching pathogenic Proteobacteria and attributed this to virus-induced type I interferons (DOI: <https://doi.org/10.1371/journal.ppat.1005572>).

In our study, vaccine-derived poliovirus sequences were co-detected in half of the specimens in which an AGE viral etiology was identified using metagenomics. The oral polio vaccine (OPV) is part of Qatar's national immunization program, which achieved 97% vaccination coverage in 2017 (Al-Dahshan et al., 2019). According to the Qatari ministry of public health, the first OPV dose is usually administered at 6 months of age, followed by two boosters at 18 months and between 4



and 6 years (<https://www.moph.gov.qa>). Poliovirus shedding is well documented among vaccinated children (Buonagurio et al., 1999; Ferreyra-Reyes et al., 2017; Troy et al., 2013). Abraham et al. revealed that poliovirus shedding is higher in children who received one or two OPV doses compared to those receiving the third dose (Abraham et al., 1993). Similarly, the highest incidence of poliovirus shedding in our study was in children up to 18 months of age. In this age group, patients were eligible to receive their first dose of oral poliovirus (OPV) vaccine according to the national vaccination program in Qatar. This suggested prolonged shedding of vaccine-related poliovirus in the stool of children.

Utilizing viral metagenomics, we were able to associate a viral etiology for at least one-third of the AGE cases that were unresolved with a conventional PCR-based assay. Due to several limitations, viral metagenomics is still considered in the development phase and is not ready yet for wide use in clinical laboratories. This includes the high cost of reagents, the need for expensive equipment, the lack of standardized protocols and user-friendly bioinformatics tools, and the complexity of the data. Additionally, maintaining updated indexes of large sequence databases remains a challenge that classification tools must address.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.meegid.2022.105367>.

## Ethical statement

The Institutional Review Boards of the American University of Beirut and Hamad Medical Corporation approved the study. Written informed consent was obtained from the children's parents to use their samples in the study.

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## Credit author statement

Ghina Hijazi: Methodology, Data curation, Formal Analysis, Writing-original draft Fatima Dakroub: Data curation, Formal Analysis, visualization, Writing- review and editing Pierre Khoueiry: Methodology, Software Abdullah El-Kurdi: Methodology, Software Amani Ezzeddine: Methodology, Formal Analysis Habib Alkalamouni: Data curation, Writing- review and editing Khalid Alansari: Conceptualization, Investigation, Funding acquisition Asmaa A Althani: Conceptualization, Data curation, Funding acquisition Shilu Mathew: Data curation, Writing-review and editing Hebah A. AlKhatib: Clinical data curation, Writing-review and editing Hadi M. Yassine: Conceptualization, Supervision, Methodology Writing- reviewing and editing, Funding acquisition Hassan Zaraket: Conceptualization, Supervision, Methodology, Formal analysis, Validation, Writing- Original draft preparation, Funding acquisition.

## Conflicts of interest

The authors declare that they have no conflicts of interest pertaining to the submitted work. The funder had no role in the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Data availability

Data will be made available on request.

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