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**Clinical and epidemiologic characteristics of Norovirus gastroenteritis among hospitalized children in Lebanon**

Melhem NM *et al.* Norovirus gastroenteritis in Lebanon

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**Abstract**

***AIM***

To assess the burden of Norovirus (NoV) and to determine the diversity of circulating strains among hospitalized children in Lebanon.

***METHODS***

Stool samples were collected from children presenting with acute gastroenteritis to 6 major hospitals in Lebanon. A total of 739 eligible stool samples, testing negative for diarrhea caused by rotavirus as a possible viral pathogen, were collected between January 2011 and June 2013. A standardized questionnaire including demographic, epidemiologic and clinical observations was used at the time of hospitalization of children presenting with diarrhea. Viral RNA was extracted from stool samples followed by reverse transcription polymerase chain reaction and nucleotide sequencing of a fragment of the viral protein 1 capsid gene. Multiple sequence alignments were carried out and phylogenetic trees were constructed using the MEGA 6 software.

***RESULTS***

Overall, 11.2 % of stool samples collected from children less than 5 years old tested positive for NoV genogroups I (GI) and II (GII). GII accounted for 10.6% of the gastroenteritis cases with only 5 samples being positive for GI (0.7%). The majority of hospitalized children showed symptoms of diarrhea, dehydration, vomiting and fever. Upon sequencing of positive samples and based on their clustering in the phylogenetic tree, 4/5 of GI-gastroenteritis cases were designated GI.3 and one case as GI.4. GII.4 was predominantly detected in stool of our study participants (68%). We report a JB-15/KOR/2008 GII.4 Apeldoorn 2008-like variant strain circulating in 2011; this strain was replaced between 2012 and 2013 by a variant sharing homology with the Sydney/NSW0514/2012/AUS GII.4 Sydney 2012 and the Sydney 2012/FRA GII.4 strains. We also report the co-circulation of non-GII.4 genotypes among hospitalized children. Our data show that NoV gastroenteritis can occur throughout the year with the highest number of cases detected during the hot months.

***CONCLUSION***

Our results are compatible with globally reported ones whereby the majority of NoV-associated viral gastroenteritis cases among our participants are attributable to GII.4.

**Key words**: Norovirus; Reverse transcription polymerase chain reaction; Sequencing; Norovirus genogroups I; Norovirus genogroups II; Lebanon

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**Core tip:** We report the results of a large scale study on Norovirus-associated gastroenteritis among children less than 5 years old in Lebanon.Our results are compatible with globally reported ones whereby the majority of viral gastroenteritis cases are attributable to GII.4. Our data support a peak incidence in July while reports from other countries show peak cases during the cold months. We report A JB-15/KOR/2008 GII.4 Apeldoorn 2008-like variant strain circulating in 2011. This strain was replaced between 2012 and 2013 by a variant sharing homology with the Sydney/NSW0514/2012/AUS GII.4 Sydney 2012 and the Sydney 2012/FRA GII.4 strains.

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**INTRODUCTION**

Gastroenteritis caused by Norovirus (NoVs) has been recently reported to be the second most common cause of acute viral gastroenteritis worldwide following rotavirus and a major cause of foodborne illness[1,2]. NoV is the leading cause of acute gastroenteritis across all age groups seeking medical care in emergency departments, outpatient clinics and the community[1]. Recent reviews of the literature on community, outpatient and hospital-based studies in developing and developed countries report that NoV gastroenteritis account for 10%-15 % of severe cases in children less than 5 years of age and 9%-15 % of mild to moderate diarrhea among individuals of all ages[3,4]. . Fecal-oral spread is the primary mode of norovirus transmission. The average incubation period is 24–48 h. The symptoms include vomiting (≥ 50% of cases), diarrhea, nausea, abdominal cramps, malaise and low-grade fever. Illness usually resolves in 12–72 h; however, it may last longer in young children, elderly people, hospitalized and immunocompromised individuals. A number of factors contribute to the high communicability of norovirus including most importantly the low infectious dose of the virus (18-100 particles), the high levels of virus shedding (> 109 particles/ml of stool during the first days after infection) known to precede illness and to be prolonged in immunosuppressed persons, the stability of the virus at temperatures ranging between 0 oC and 60 oC, and finally the high rate of mutation and recombination leading to antigenic diversity[5-7]. While 75% of NoV cases have been reported during the cooler month, geographic variability and annual fluctuations have also been described[8].

NoVs are non-enveloped, polyadenylated, single stranded, positive-sense RNA viruses of the family *Caliciviridae.* The RNA genome of NoVs is composed of 3 large open reading frames (ORFs) designated as ORF-1, ORF-2 and ORF-3. ORF-1 encodes 6 non-structural proteins including the protease and the RNA-dependent RNA-polymerase (RdRp). ORF-2 and ORF-3 encode the structural viral components viral protein 1 (VP1) (major capsid protein) and VP2 (minor capsid protein), respectively. Based on the amino-acid sequence of VP1, noroviruses are divided into 6 genogroups (GI-GVI). GI, GII and GIV are known to infect humans[9,10]. Genogroups are further subdivided into genotypes based on the RdRp sequence or capsid sequence. At the genomic level, strains of the same genogroups are 51%-56% similar whereas genotypes have 69-87% similarity[11,12]. At least 8 and 21 genotypes belong to GI and GII, respectively[1]. The genogroup II, genotype 4 noroviruses, designated GII.4, are responsible for the majority of NoV outbreaks in the United States, Australia and many European countries[13,14]. GII.4 NoVs are continuously changing and viral variants emerge every couple of years every 2-7 years as a result of genetic drift, an observation compatible with the immune escape mechanism observed with influenza A virus[15-19]. Globally and during the past decade, GII.3 and GII.6 were reported as second and third predominant genotypes after GII.4, respectively[13].

To our knowledge, there are no large scale studies conducted in Lebanon on NoV and its association with acute gastroenteritis. The aim of this study is to determine the prevalence of Norovirus gastroenteritis as well as the genotypic characterization of the virus among hospitalized children less than 5 years old.

**MATERIALS AND METHODS**

***Study population and specimen collection***

The study was conducted in accordance with the ethical guidelines of Helsinki and after approval of the Institution Review Board of the American University of Beirut. Written informed consents were obtained from the legal guardians of hospitalized children and consequently stool samples and medical data were collected. A standardized questionnaire including demographic, epidemiologic and clinical observations was used at the time of hospitalization of children presenting with diarrhea. Stool samples were collected from children presenting with acute gastroenteritis to 6 major hospitals in Lebanon. A total of 739 eligible stool samples, testing negative for diarrhea caused by rotavirus as a possible viral pathogen, were collected over a two-year period (January 2011- June 2013).

***Viral RNA extraction and NoV detection***

0.5-1.0 mL of collected stool was suspended in 5 mL of 0.89% NaCl. The fecal suspension was centrifuged following which the supernatant was filtered and 140 μL of the filtrate used for viral RNA extraction. QIAamp Viral RNA Mini Kit (Qiagen, Germany) was used for viral RNA extraction according to the manufacturer’s instructions. Viral RNA was stored at -20 oC.

***PCR and sequencing***

Reverse transcription polymerase chain reaction (RT-PCR) was performed using genogroup-specific primers as previously described[20-22]. Briefly, RT-PCR targeted the 5’ end of the capsid region in ORF2 using: G1-SKF (+CTG CCC GAA TTY GTA AATGA) and G1-SKR (-CCA ACC CAR CCA TTR TACA and primers GoG2F (+CAR GAR BCN ATG TTY AGR TGG ATGAG) and G2-SKR (-CCR CCN GCA TRH CCR TTR TACAT) for amplifying a 330 bp and a 387 bp PCR products of GI and GII genogroups, respectively. QIAGEN OneStep RT-PCR Kit (Qiagen, Germany) was used under the following conditions:42٥ C for 30 min; initial PCR activation step at 95 ٥C for 15 min; denaturation at 94٥ C for 30 s, annealing at 52-54 oC for 30 s, extension at 72 oC for 45 s (30 cycles); and final extension at 72 oC for 7 min. Synthetic Norovirus G1 (I) RNA (ATCC® VR3199SD™) and Synthetic Norovirus G2 (II) RNA (ATCC® VR3200SD™) were used as positive controls. The PCR products were analyzed by gel electrophoresis and stored at -20 oC until analysis. Nucleotide sequencing of NoV positive samples was performed by Macrogen, Inc. (Seoul, South Korea) using the PCR primers. A total of 19 full length human NoV capsid protein sequences were downloaded from GenBank and used as reference strains. These included 6 GI and 16 GII with the following accession numbers : AAS86780.1 (GI), ACN32270.1 (GI.1), ACU56258.1 (GI.2), ACX33982.1 (GI.3), ACV41096.1 (GI.4), ADB54834.1(GI.8), aAIO11150.1 (GII), ABC96332.1 (GII)**,** AFA55174.1 (GII.1), BAG68716.1 (GII.2), ADK23787.1 (GII.3), AEG79292.1 (GII.4), ABL74397.1 (GII.4), ABL74391.1 (GII.4), AGT95930.1 (GII.4), KM245069.1 (GII.4 Yerseke/2006a), KF361437.1 (GII.4 Minerva/2006b), KP762437.1 (GII.4 Den Haag 2006b), ADE28721.1 (GII.6), ACX85810.1 (GII.7), ADZ24003.1 (GII.12), and ACX81355.1 (GII.14. Multiple sequence alignments were carried out using CLUSTALL or BioEdit out and phylogenetic trees were constructed using the MEGA 6 software. The phylogenetic tree was generated using the neighbor-joining method validated by 1000 bootstrap replicates.

***Nucleotide sequence accession numbers***

The partial nucleotide sequences determined in this study were deposited in GenBank with the following accession numbers: GI KU950315- KU950319 and GII KU963412 - KU963487.

***Statistical analysis***

Data was analyzed using SPSS Statistics version 22. For comparisons of demographic and clinical symptoms, chi-square analysis and Pearson χ2 were used.

**RESULTS**

In this study, a total of 739 eligible rotavirus-negative stool samples were assayed for NoV by RT-PCR during the 2-years study period (January 2011-June 2013). Stoolsamples were collected from children less than 5 years presenting to six hospitals in Lebanon due to acute gastroenteritis. Tables 1 and 2 summarize the demographic and clinical characteristics of our study participants. Overall, 11.26% (*n =* 83/739) of the samples tested positive for NoV (Table 1). The majority of cases were NoV genogroup GII (*n =* 78/83) (Table 2) with a total incidence rate of 10.6%, while only 5 samples tested positive for NoV genogroup GI with a total incidence rate of 0.7% . We did not have mixed infections with NoV GI and GII among our study participants. Males accounted for 55.9% (413/739) of hospitalized children and females for 44% (326/739). 11.4 % of the males study participants and 11% of females participants were NoV-positive (Table 1). Being a male or a female was not significantly associated with NoV infection (*P* = 0.887). The mean age of the study participants testing positive for NoV and presenting with gastroenteritis symptoms upon admission was 16.2 ± 9.5 mo. 15.5% of samples testing positive for NoV and presenting to hospitals with acute gastroenteritis symptoms were children of 12-23 months of age (35/376) followed by children of 24-35 months of age (12.7% ; 10/79) (Table 1). Our results show however, that there is no association between age and NoV infection among our study participants (*P* = 0.729).

As expected, the majority of our study participants testing positive for NoV had symptoms of diarrhea (95%), dehydration (90%), vomiting (76%) and fever (67.5%). The Vesikari Clinical Severity Scoring System was used to assess the severity of acute gastroenteritis. Severe gastroenteritis (*i.e.*, a score above 11) was reported in 92% of NoV-positive participants (Table 2). The average hospital stay of children admitted ranged between 3 and 5 days. 95% of NoV-positive cases received intravenous IV rehydration whereas only 18% received oral rehydration during hospitalization.

NoV incidence was similar across different geographic regions. Incidences in hospitalized children were 9%, 13% and 11% in Beirut, the Southern and the Northern parts of Lebanon, respectively (*P* = 0.371). Overall 11.23% (83/739) tested positive for NoV among our study participants of which 45 (54%) were detected in 2011 and 36 (43%) detected in 2012 and two samples tested positive testing during the first half of 2013. The seasonal onset of NoV cases was similar during 2011 and 2012 as described in Figure 1. While our data show that NoV infection can occur throughout the year, the highest percentage of NoV-positive samples was detected in July 2011 (24%) and July 2012 (27%), i.e. in the hot months. Fewer infections were observed between October and February which are the cooler months in Lebanon.

In order to analyze the extent of the genetic diversity and to designate the genotypes of NoVs detected among our cohort of children less than 5 years old, we inferred the phylogenetic relationship of the major capsid protein gene (*orf2)* along with subgenotype reference isolates. We sequenced a total of 81 samples rather than the total number of NoV-positive samples (*n =* 83) due to the lack of sufficient volume of purified RNA for two samples. Among GI samples, 4/5 were designated GI.3 and 1/5 as GI.4 based on their clustering in the phylogenetic tree. A total of 8 different NoV GII genotypes were detected among our study participants whereby 68% (52/76) of positive cases were attributed to GII.4. GII.4 diversified into two distinct subclusters distinguished by an A151T substitution. These subclusters co-circulated between 2011 and 2013 (Figure 2). While GII.4 was predominantly associated with gastroenteritis among our study participants, circulation of more than one subgenotype during the same year was also recorded. The following non-GII.4 genotypes were also detected among hospitalized children during the study period: GII.6 (7/76, 9.2%), GII.21 (5/76, 6.6%), GII.3 (5/76, 6.6%), GII.13 (3/76, 3.95), GII.9 (1/76, 1.3%), GII.1 (1/76, 1.3%), and GII.2 (1/76, 1.3%) (Figure 2).

**DISCUSSION**

We report the results of a large-scale study on NoV-associated gastroenteritis in Lebanon.Our study reflects the predominance of GII strains among children less than 5 years of age hospitalized due to acute gastroenteritis. We detected a broad genetic diversity of noroviruses causing acute gastroenteritis among our study participants. Overall, GII.4 (68%) was the most prevalent genotype isolated from hospitalized children in Lebanon during the study period Our results are compatible with globally reported ones whereby the majority of NoV-associated viral gastroenteritis cases are attributable to GII.4[23-25], and co-circulating with other genotypes[13,26-28]. Locally, NoV GII has been previously reported in 5 Lebanese children less than 10 years old[29]. Regionally, in the Middle East and North Africa (MENA), a number of studies have recently assessed the prevalence of NoV among hospitalized children less than 5 years old (hospitalized due to signs of acute gastroenteritis). These studies were performed on a variable sample size in Egypt[30], Israel[31,32], Iran[33], Jordan[34], Kuwait[35], Libya[36,37], Morocco[38], Tunisia[39,40], Turkey[41-45] and Yemen[46]. NoV was detected in stool samples of 6%-30% of hospitalized children less than 5 years with GII.4 and GII.3 predominantly reported in these studies.

We report A JB-15/KOR/2008 GII.4 Apeldoorn 2008-like variant strain circulating in 2011 among children less than 5 years in Lebanon.This strain was replaced between 2012 and 2013 by a variant sharing homology with the Sydney/NSW0514/2012/AUS GII.4 Sydney 2012 and the Sydney 2012/FRA GII.4 strains. The latter emerged in Australia in March 2012 and was later isolated from the United States, Belgium, Denmark, Scotland, and Japan[47]. The co-circulation of several GII.4 lineages is well described[18,48] and has been suggested to be a mechanism of positive selection of mutations to generate new NoV variants[49]. The variants of the NoV GII.4 lineage have been associated with 62% to 80% of NoV gastroenteritis worldwide, as well as explosive outbreaks occurring in community settings[11,50]. Global epidemics of NoV-gastroenteritis have been associated with the following viral strains: US 1995/96 in 1996[51], Farmington Hills in 2002[52,53], Hunter in 2004[54], 2006b virus in 2007 and 2008[55], New Orleans virus during 2009-2012[56] and Sydney 2012[57]. Other GII.4 variants have also been associated with localized types of epidemics such as Henry 2001, Japan 2001, Asia 2003, and 2006a and Apeldoorn 2008[18].

While GII.3 was reported to be the second predominant genotype in many countries, it ranked third along with GII.21 among our study participants after GII.4 and GII.6. GII.6 and GII.2 are reported to account for 5% of the globally reported strains. The prevalence of GII.6, the second predominant cause of gastroenteritis among our study participants, was similar to what has been reported in several countries including Brazil[58], Japan[59], Africa[60] and Finland[61]. GII.21, previously reported in Brazil[62] has been described as a recombinant product between GII.4/2006b and GII.18 strains[63]. In our study this genotype is similar to the Salisbury150/2011/USA GII.21. GII.13, previously described as an uncommon cause of gastroenteritis, is increasingly being reported in many Asian countries[13]. Our results show that GII.13 ranked forth as a causative agent of gastroenteritis among hospitalized children in Lebanon. Among GI, GI.3 was predominantly detected albeit less often compared to GII. Our results are compatible with globally reported ones whereby GII is the most prevalent genogroup causing approximately 96% of infections with GI constituting the remaining genogroup and causing on average 3.6% of noroviral infections[13].

Reports show that NoV cases peak during the cold months in parts of Europe and North America with sporadic cases detected all year round as well as outbreaks during the summer time[59,64-66]. Similar results are reported from few other countries[33,42,43,67]. New GII.4 variants are reported to emerge with unusual spring/summer seasonality[52] along with a total increase in wintertime disease in Europe. The former was suggested to be due to the lack of an effective immunity to the newly emergent GII.4 variant. Recently, a systematic review reported a variable global seasonality of NoV[8]. A peak in winter months was reported in the Northern Hemisphere while peak cases and outbreaks were reported in the hot months in the Souther hemisphere. Little data exist from Africa, the tropical regions and the MENA region and when existing, these studies report on NoV detected in diverse settings and of short duration. Consequently, the ability to associate climate and demographic factors on the seasonality of NoV in these areas is difficult. While our data supports a peak incidence in July, the seasonal pattern of NoV infection in Lebanon is to be further investigated in comparison with existing reports. In summary, the genotypic characterization of NoV positive samples revealed a wide diversity of circulating genotypes with GII.4 predominantly being associated with gastroenteritis. Globally, GII.4 circulating strains have been responsible of large number of outbreaks in many countries including China, India, Japan, Egypt, Turkey and Italy[13]. The evolution of GII.4, described as being epochal (long periods of status quo followed by outbursts of variation) over time generates escape mutants that are periodically selected for by herd immunity[11]. Further studies are needed to assess the extent of NoV molecular diversity in Lebanon among different age groups.

While we partially genotyped our circulating strains*,* we realize that we cannot suggest any recombination event to elaborate on the diverse genotypes detected among our study participants. Nevertheless, our study confirms the significant role of NoV as a causative agent of gastroenteritis among children less than 5 years old in Lebanon. Our results are compatible with globally reported ones whereby the majority of NoV-associated viral gastroenteritis cases among our participants are attributable to GII.4. The continuous monitoring of NoV infection among different age groups is needed in Lebanon to support intervention strategies and to detect new circulating variants possibly associated with increased rates of morbidity.

In conclusion, we report the results of a large-scale study on NoV-associated gastroenteritis in Lebanon. Our results are compatible with globally reported ones whereby the majority of viral gastroenteritis among children less than 5 years old are attributable to GII.4. Moreover, our data support a peak incidence in July whereas other reports show peak incidences during the cold months (*e.g*., North America, parts of Europe). The seasonal pattern of NoV in Lebanon should be further investigated. Efforts should be made to introduce the clinical diagnosis of the virus due to its impact on the community as well as health care institutions.

**COMMENTS**

***Background***

Norovirus (NoV) is one of the most common causes of acute gastroenteritis among children. To our knowledge, there are no large scale studies conducted in Lebanon on NoV and its association with acute gastroenteritis among children less than 5 years old. Moreover, the authors have no data on the genotypic characterization of the predominantly circulating NoV strains in Lebanon as compared to other countries. This study is important to support intervention strategies.

***Research frontiers***

NoV is the leading cause of acute gastroenteritis across all age groups seeking medical care in emergency departments, outpatient clinics and the community. Recent reviews of the literature on community, outpatient and hospital-based studies in developing and developed countries report that NoV gastroenteritis account for 10%-15% of severe cases in children less than 5 years of age and 9%-15% of mild to moderate diarrhea among individuals of all ages. Our data are compatible with globally reported ones whereby NoV Genogroup 2 genotype 4 (GII.4) is the most prevalent strains associated with gastroenteritis.

***Innovations and breakthroughs***

To the knowledge of the authors, there are no large scale studies conducted in Lebanon on NoV and its association with acute gastroenteritis. The aim of this study is to determine the prevalence of Norovirus gastroenteritis as well as the genotypic characterization of the virus among hospitalized children less than 5 years old. This study is the first in Lebanon to report on the circulating strains of NoV GI and GII among children hospitalized due to acute gastroenteritis.

***Applications***

This study is the first to report on the clinical epidemiology, seasonality and genotypic characterization of NoV as a causative agent of acute gastroenteritis leading to hospitalization among children less than 5 years old in Lebanon. This study is important to guide intervention strategies in Lebanon as well as the national introduction of clinical diagnosis of the virus as a major cause of gastroenteritis.

***Peer-review***

Important work on an interesting topic, the clinical and epidemiologic characteristics of norovirus gastroenteritis among hospitalized children in Lebanon.

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**Figure 1 Seasonal distribution of Norovirus cases among children less than 5 years old in Lebanon.** The absolute number (left Y-axis) and the the percentage (right Y-axis) of Norovirus (NoV)-positive cases isolated from stool samples and collected from children presenting with acute gastroenteritis to six major hospitals in Lebanon are depicted against month and year of circulation (X-axis).



**Figure 2 Phylogenetic analysis of the norovirus VP1 major capsid gene.** Nucleotide sequences spanning nucleotides 5385-5657 (length = 273 nt) of NoV isolated in Lebanon were aligned with reference strains obtained from GenBank. The trees were then constructed based on the nucleotide sequences using the Neighbor-Joining method with bootstrap analysis of 1,000 replicates using MEGA 6.0. Bootstrap values greater than 70% are shown. Reference strains are in bold. LBM: Lebanon Makassed Hopsital; LBH: Lebanon, Hammoud Hospital; LBN: Lebanon, Nini Hospital; LBR: Lebanon, Hariri Governmental Hospital; LBA: Lebanon, American University of Beirut Medical Center; LBNG: Lebanon, Nabatiyeh Hospital.

**Table 1 Demographic characteristics of study participants *n* (%)**

|  |  |  |  |
| --- | --- | --- | --- |
|  | ***n*** | **NoV positive** | **NoV negative** |
|  |  |  |  |
| Participants | 739 | 83 (11.2) | 656 (88.8) |
| Gender |  |  |  |
| Male | 413 | 47 (11.4) | 366 (88.6) |
| Female | 326 | 36 (11.0) | 290 (89.0) |
| Age group |  |  |  |
| 0-11 | 376 | 34 (9.0) | 342 (91.0) |
| 12-23 | 226 | 35 (15.5) | 191 (84.5) |
| 24-35 | 79 | 10 (12.7) | 69 (87.3) |
| 36-47 | 30 | 3 (10.0) | 27 (90.0) |
| 48-59 | 27 | 1 (3.7) | 26 (96.3) |
| Region |  |  |  |
| Beirut | 217 | 20 (9.2) | 197 (90.8) |
| North Lebanon | 315 | 35 (11.1) | 280 (88.9) |
| South Lebanon | 207 | 28 (13.5) | 179 (86.5) |
| NoV: Norovirus. |  |  |  |

**Table 2 Clinical characteristics of Norovirus-positive cases, *n* (%)**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **NoV positive** | **GI** | **GII** |
| Fever |  |  |  |
| Yes | 56 (67.5) | 5 (100.0) | 51 (65.4) |
| No | 27 (32.5) | 0 (0.0) | 27 (34.6) |
| Vomiting |  |  |  |
| Yes | 63 (75.9) | 3 (60.0) | 60 (76.9) |
| No | 20 (24.1) | 2 (40.0) | 18 (23.1) |
| Diarrhea |  |  |  |
| Yes | 79 (95.2) | 5 (100.0) | 74 (94.9) |
| No | 4 (4.8) | 0 (0.0) | 4 (5.1) |
| Assessed Dehydration |  |  |  |
| Severe | 10 (12.0) | 1 (20.0) | 9 (11.5) |
| Mild to moderate | 64 (77.1) | 3 (60.0) | 61 (78.2) |
| No dehydration | 9 (10.8) | 1 (20.0) | 8 (10.3) |
| Vesikari Score |  |  |  |
| Severe | 76 (91.6) | 4 (80.0) | 72 (92.3) |
| Mild to moderate | 7 (8.4) | 1 (20.0) | 6 (7.7) |

NoV: Norovirus.