Aetiology of acute paediatric gastroenteritis in Bulgaria during summer months: prevalence of viral infections

Zornitsa Mladenova,1 Andrej Steyer,2 Adela Fratnik Steyer,3 Balasubramanian Ganesh,4† Petar Petrov,5 Tanja Tchervenjakova6 and Miren Iturriza-Gomara7

1 (former) Department of Virology, National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria
2University of Ljubljana, Faculty of Medicine, Institute of Microbiology and Immunology, Ljubljana, Slovenia
3Blood Transfusion Centre of Slovenia, Ljubljana, Slovenia
4Division of Virology, National Institute of Cholera and Enteric Diseases, Kolkata, India
5Infectious Ward, University Hospital ‘St Anna’, Sofia, Bulgaria
6Infectious Wards 1 and 2, Specialized Hospital for Infectious and Parasitic Diseases ‘Prof. Ivan Kirov’, Sofia, Bulgaria
7Institute of Infection and Global Health, University of Liverpool, Liverpool, UK

Paediatric acute gastroenteritis is a global public health problem. Comprehensive laboratory investigation for viral, bacterial and parasitic agents is helpful for improving management of acute gastroenteritis in health care settings and for monitoring and controlling the spread of these infections. Our study aimed to investigate the role of various pathogens in infantile diarrhoea in Bulgaria outside the classical winter epidemics of rotavirus and norovirus. Stool samples from 115 hospitalized children aged 0–3 years collected during summer months were tested for presence of 14 infectious agents – group A rotavirus, astrovirus, Giardia, Cryptosporidium and Entamoeba using ELISAs; norovirus by real-time RT-PCR; picobirnavirus and sapovirus by RT-PCR; adenovirus using PCR, and Salmonella, Shigella, Escherichia coli, Yersinia and Campylobacter using standard bacterial cultures. Infectious origin was established in a total of 92 cases and 23 samples remained negative. A single pathogen was found in 67 stools, of which rotaviruses were the most prevalent (56.7 %), followed by noroviruses (19.4 %), enteric adenoviruses (7.5 %), astroviruses (6.0 %), bacteria and parasites (4.5 % each) and sapoviruses (1.4 %). Rotavirus predominant genotypes were G4P[8] (46.3 %) and G2P[4] (21.4 %); for astroviruses, type 1a was the most common, while the GII.4/2006b variant was the most prevalent among noroviruses. Bacteria were observed in five cases, with Salmonella sp. as the most prevalent, while parasites were found in ten stool samples, with Giardia intestinalis in five cases. The results demonstrated high morbidity associated with viral infections and that rotavirus and norovirus remain the most common pathogens associated with severe gastroenteritis during summer months in Bulgaria, a country with a temperate climate, and significant molecular diversity among circulating virus strains.

INTRODUCTION

Paediatric acute gastroenteritis (AGE) is a global public health problem accounting for 1.5 billion diarrhoeal episodes and 3 million deaths each year. A broad spectrum of enteric pathogens can cause acute infantile diarrhoea (Hilmarsdottir et al., 2012; González et al., 2011; Tam et al., 2012). The main bacterial and protozoan organisms isolated
from stool samples of children less than 5 years of age are diarrhoeagenic Escherichia coli, Salmonella spp., Shigella spp., Yersinia spp. and Campylobacter spp., and Giardia intestinalis, Entamoeba histolytica and Cryptosporidium spp., respectively. In addition, the representatives of four viral families – rotaviruses (RVs) (Reoviridae), noroviruses (NoVs) and sapoviruses (SaVs) (Caliciviridae), human astroviruses (AstVs) (Astroviridae) and adenoviruses (AdVs) subgenus F (Adenoviridae) – are commonly detected in childhood viral gastroenteritis (González et al., 2011; Tam et al., 2012).

Human RV group A is considered the leading cause of AGE in children less than 5 years, and is responsible for over 140 million diarrhoeal episodes yearly worldwide. RV is a non-enveloped virus with a genome of 11 double-stranded RNA segments and triple-layered capsid with two surface proteins: VP7 (glycoprotein, G) and VP4 (protease-sensitive protein, P). RVs are highly divergent and, to date, 27 G and 35 P types have been described (Matthijssens et al., 2011). NoVs affect individuals of all ages, and are the most common cause of sporadic cases and outbreaks of gastroenteritis (Tam et al., 2012; Ahmed et al., 2014), while SaVs lead to relatively mild gastroenteritis in small outbreaks and sporadic cases. These viruses are non-enveloped, with an icosahedral capsid that surrounds a positive-sense single-stranded RNA genome with three overlapping ORFs encoding the non-structural (ORF1) and structural (ORF2 and ORF3) viral proteins. Based on their genetic heterogeneity, NoVs are currently classified in six genogroups (GI–GVI), with GI (nine genotypes) and GII (22 genotypes) containing the majority of the strains associated with human disease (Green, 2013). Phylogenetically, SaVs can be divided into five genetic clusters, GI–GVI, based on their capsid-coding region, and except for GIII, all other genotypes infect humans (Hansman et al., 2007). AstVs and enteric AdVs (types 40 and 41) are much less often identified as cause of acute diarrhoea in children under the age of 5 years. The incidence of AstV infections ranges from 2–9 % in developed countries up to 26 % in developing countries. Eight AstV genotypes and 17 lineages have been identified (Martella et al., 2014), with genotype 1 being the predominant type in most parts of the world (Mendéz & Arias, 2007). Among AdVs, subgroup F has been detected in AGE cases most frequently, but other subgenera such as A (type 31) and C (type 2) have also been implicated in infantile diarrhoea. Moreover, some other viral agents such as picobirnaviruses (PBVs, Picobirnaviridae), Aichi virus and parechoviruses (Parechoviridae) and toroviruses (Coronaviridae) have also been reported in association with human gastroenteritis (Garrino et al., 2008).

In Bulgaria, all hospitalized cases of infectious AGE are notifiable in the National Notifiable Disease Surveillance System (www.ncipd.org). Infectious diarrhoea is defined as the presence of three or more defecations per day with stools with changed consistency, colour, smell and pathological impurities (mucus/blood), and when one or more of the following criteria are met: fever, vomiting, abdominal cramps, epidemiological background (recent travel history, consumption of food/drinks, attendance at water sources, etc.). Indications for hospital admission are patient’s age (infants and toddlers <1 year old, adults >60 years old) and health conditions (moderate to severe clinical conditions, prematurity, additional acute/chronic disease). The investigation of enteropathogenic bacteria, Salmonella spp., Shigella spp., diarrhoeagenic E. coli, Yersinia spp. and Campylobacter spp. are obligatory, as well as for RVA in children <9 years of age (since June 2011).

Currently, there are no published data on the aetiology of paediatric acute diarrhoea in Bulgaria and, in the absence of routine screening, the incidence of virus infections other than RV remains largely unknown.

Increased morbidity associated with AGE of unknown aetiology during summer months has been observed in Bulgaria since 2007 (www.ncipd.org). In order to investigate the spectrum of diarrhoeal enteropathogens (six viral, five bacterial and three parasitic) and to assess molecular epidemiology of the main viral agents, we conducted a study including 115 children with AGE symptoms aged 0–3 years, hospitalized in the infectious wards of two metropolitan hospitals in the summer of 2009.

**METHODS**

**Patients and samples.** Our study was conducted at two metropolitan hospitals: the Specialized Hospital for Infectious and Parasitic Diseases 'Prof. Ivan Kirov' and the University Multi-Profiled Hospital for Active Treatment 'St Anna', Sofia, Bulgaria. These hospitals have the only paediatric infectious wards that serve the child population in Sofia city and region of approximately 40 000–42 000 children aged 0–5 years (data of the National Statistic Institute for 2013).

A total of 115 children hospitalized with AGE between June and September 2009 were randomly selected and enrolled in the study. The children were between 40 days and 3 years old, and the male:female ratio was 1:1. The study population represented 10 % of all children <3 years old admitted to both hospitals with AGE for the entire period of the study.

The criteria for hospital admission of children with AGE were presence of diarrhoea (three or more watery or loose stools in the preceding 24 h) and/or vomiting (two or more episodes in 24 h) and/or fever (>37 °C) and clinical evidence of grade II–III dehydration (>5 % body weight loss, abnormal skin turgor and elasticity, sunken eyes and/or fontanelles, changes in pulse/heart and respiratory rate, mental changes such as lethargy or irritability, decreased urine volume). Children with prolonged diarrhoea (>1 week) and those who received antibiotic therapy before admission were excluded from the study. On admission all patients were clinically examined and the patient’s history including nutritional and epidemiological information was documented.

One stool sample was collected from each child and was divided into three portions: one for bacterial testing, the second for viral and protozoal detection and the third portion was stored at −70 °C for molecular-based investigation.

**Laboratory tests and analysis.** Bacterial pathogens such as Salmonella spp., Shigella spp., diarrhoeagenic E. coli, Yersinia spp. and Campylobacter spp. were detected using standard bacterial
cultures. Presence of group A RVs, AstVs, G. intestinalis, Cryptosporidium parvum and Cryptosporidium hominis, and Entamoeba histolytica and Entamoeba dispar were examined using antigen-detection immunoenzymne commercial tests RIDASCREEN (R-Biopharm). All stools were prepared as 20% stool suspension in 1 ml PBS and viral RNA/DNA was extracted by PureLink Viral RNA/DNA Mini kit (Invitrogen) according to manufacturer’s instructions. Extracted RNA/DNA was eluted in 50 μl, and 20 μl of eluent was reverse-transcribed using random hexamers (Invitrogen). RV-positive samples were characterized by semi-nested PCR with primers toward VP7 and VP4 genome segments of group A RVs according to European Rotavirus Detection and Characterization Methods v4 (http://www.eurotota.net/docs.php; Iturrizá-Gómara et al., 2011). Previously described oligonucleotide primer sets Mon340/Mon348, targeting the ORF1a (Belliot et al., 1997), and Mon269/Mon270, targeting ORF2 (Noel et al., 1995), were used to obtain initial PCR products for sequencing and genotyping of the AstV strains detected. Real-time RT-PCR for NoV genogroups I and II detection was performed with primers COG1F-COG1R and COG2F-COG2R and FAM-BHQ-labelled fluorescent probe mixture of RING1(a)-TP and RING1(b)-TP, and RING2-TP, respectively, using Opticon 2 real-time cycler (Bio-Rad) (Kojima et al., 2003). In addition, a total of eight PCR products of NoVs GI (two strains) and GII (six strains) were obtained after amplification by primer sets toward the capsid-coding region (Kojima et al., 2002). The presence of SaV and of AdV group F was detected by PCRs using primer pairs SR80-JV33 and AdеноF-AdеноR, and protocols described previously (Vinje et al., 2000; Tiemessen & Nel, 1996). In addition, extracted RNAs were tested for the presence of PBV genogroups I and II, as described elsewhere (Bhattacharya et al., 2006). A detailed genetic characterization of a total of 16 viral strains (seven AstVs, two NoVs GI, six NoVs GII and one SaV) was performed by direct sequencing, using ABI PRISM BigDye Terminator Cycle Sequencing Reaction kit (Applied Biosystems). The sequences obtained were edited manually and aligned using the BLAST program. The nucleotide comparisons were done by BioEdit and MEGA4 software, and phylogenetic trees were reconstructed using the neighbour-joining method and bootstrap 100 through the MEGA4 program (Tamura et al., 2007). Nucleotide sequences of Bulgarian NoV, SaV and AstV strains obtained in the present study have been deposited in GenBank under the accession numbers KP123636–KP123644 and KP162248–KP162261.

RESULTS

Infectious origin of AGE was established in 92 of 115 cases (80%) and 23 (20%) samples remained negative after all diagnostic tests were performed. A single pathogen was found in 67 stools, of which RVs were the most prevalent (38; 56.7%), followed by NoVs (13; 19.4%), enteric AdVs (5; 7.5%), AstVs (4; 6%), bacteria (3; 4.5%), parasites (3; 4.5%) and SaVs (1; 1.5%). Double and triple infections were detected in 19 (16.5%) and six (5.2%) of samples, respectively, mostly combinations of RV or NoV with other pathogens (Table 1). PBVs genogroups I and II were not detected in the stool specimens tested.

Diarrhoea severity was recorded from all children enrolled in the study. The mean duration of diarrhoea was shortest in viral infections (3.3 ± 0.48 days) and longest in bacterial infections (9.8 ± 1.6 days). Vomiting of >3 episodes/day⁻¹ was commonly registered among patients with NoVs (90%) and RVs (76%), while high temperatures >38°C were mostly associated with bacterial AGE (92%). Upper respiratory tract infections were most common in RV (37%) and enteric AdV (32%) groups, whereas mucoid/bloody stools and abdominal cramps were found in the majority of patients (81%) with bacterial infection. No differences were found on the severity of disease between mixed viral–bacterial infections and bacterial alone. All patients fully recovered and were discharged.

Overall, RVs were found in a total of 56 samples (48.7%), among which genotypes G1P[8] (3; 5.4%), G2P[4] (12; 21.4%), G4P[8] (26; 46.3%) and G9P[8] (7; 12%) were detected most frequently. Unusual G–P combinations such as G12P[8] and G4P[6] (1; 1.8% each) were also identified. Co-infections with two RV strains, G4P[8] and G3P[8], were observed in two stool samples and four RV strains remained partly genotyped (Table 2).

NoV genogroups I and II were detected in 15 (13%) and 18 (15.7%) samples, respectively, using real-time RT-PCR. Five GI and six GII NoV-positive samples were selected based on their different collection points for performing conventional RT-PCR followed by sequencing. Of these, amplicons of two GI isolates and all six GII NoVs were successfully obtained. Part of the ORF2, the capsid-coding fragment of viral genome, of the two GI Bulgarian NoVs was sequenced, and the phylogenetic analysis allowed their clustering into genotype GI.4 (Fig. 1). The GI NoV strains

| Table 1. Viral, bacterial and parasitic agents detected in single, double and triple infections in 115 sporadic AGE cases in children aged 0–3 years old |
|-----------------|-----------------|-----------------|-----------------|
| **Single infections** | **Total** | **Rotavirus group A** | **Norovirus** |
| **Double infections** | **19** | **9** | **3** |
| **Triple infections** | **6** | **2** | **1** |
| **Total** | **67** | **38** | **13** | **4** | **5** | **1** | **3** | **1** |

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*Note: G – genotypes, P – phenotypes.*
BG629/2009 and BG675/2009 had nucleotide (nt) and amino acid (aa) sequence homology of 91 and 95 %, respectively. The strain BG629/2009 was 98–99 % nt similar to several NoVs isolates from oysters in Taiwan in 2009 (GQ401126-129), while isolate BG675/2009 resembled the US strain Hu/GL4/1643/2008/US (GQ413970) and NoVs detected in a water-borne outbreak in Sweden (Nenonen et al., 2012).

Among the GII NoVs investigated in the present study, all strains but one belonged to genotype GII.4. A single strain, BG638/2009, was closely related to the GII.3 NoV strains (Fig. 2). Phylogenetic analysis of the capsid-coding region showed that it had 96–97 % nt homology with the GII.3 strain Hu/5017.34/2003/JPN (EU187437), detected in Japan in 2003–2004, and the recombinant Hu/GL4/CBNU1/2006/KOR (GU980585) from an outbreak in South Korea 3 years later. The genetic analysis of part of the ORF1 gene of BG638/2009 revealed it was GIIb genotype. All five GII.4 NoVs (BG672/2009, BG703/2009, BG737/2009, BG746/2009 and BG752/2009) represented the pandemic GII.4 DenHaag 2006b variant (EF126965) according to the capsid gene, and displayed 98–99 % nt and 99–100 % aa inter-strain similarity.

A single SaV strain was detected in the present study (prevalence of 0.9 %). The sequence and phylogenetic analyses of partial RNA-dependent RNA polymerase (RdRp) gene revealed that Bulgarian strain BG697/2009 shared closest match (95 % nt identity) to SaV strains detected in Turkey in 2007 (GQ253120), in India in 2007 (AB447416) and in different parts of the world during the past decade (Fig. 3). The BG679/2009 strain showed also 90–93 % nt homology to other strains included in the GII genetic cluster –, Ehime-1107 (DQ058829) and SW278 (DQ125333), which were described as inter-genogroup recombinant SaV strains (Hansman et al., 2007).

Seven of eight AstV strains detected in this study were analysed by sequencing of fragments of ORF1a (nt 1248–1467; 219 nt length) and ORF2 (nt 4579–4959; 380 nt length) genes (Fig. 4a, b). Phylogenetic analysis of Bulgarian AstV strains revealed that four of the strains (BG668, BG710, BG741, BG747) aligned (99–100 % homology) with a type 1a strain, MAstV/Hu/HUN/2010/Nyergesujufalu/HUN4520 (HQ398856), that was detected in 2010 (1 year later) and was responsible for a single outbreak in a nursery in Hungary. The ORF1a and ORF2 sequences of the Bulgarian AstV BG703 were found to be 99 and 100 % similar at nt and aa levels to the strain MAstV/Hu/DEU/2004/Dresden-1 (AY720892), which belongs to the type 1d. The genetic analysis of AstV BG704 showed that it closely matched unassigned isolates from India V1182 (AB325804) and Korea (clones from KS106205 to KS106208; AF361030 to AF361033) in ORF1a, while the ORF2 part was 99 % nt and 100 % aa similar to several Pakistani type 1 strains (reference strain PAKNIH-3075; KC896126). In addition, BG strain BG656 clustered in a common branch with strain MAstV/Hu/PAK/2008/PAK_NIH_VS_908, a type 3/5 recombinant from Pakistan, and type 5 Chinese AstV DL030 (IJO3408) according to the ORF1a sequence, but it was 100 % nt and aa similar to the type 3a strain isolated in Russia in 2008, RUSnc08-3364, based on the ORF2 sequence.

Bacterial pathogens were found in five diarrhoeal cases (4.3 %), with Salmonella species being the most common (2.6 %). Strains of Salmonella enteridis groups OC and OD were identified in two and one faecal samples, respectively. Enterohaemorrhagic E. coli strain O157 and Shigella flexneri strains were detected as co-pathogens in single samples (0.9 % each). Parasites were found in ten stool samples (8.7 %) with G. intestinalis in five samples (4.3 %), Cryptosporidium spp. in three samples (2.6 %) and Entamoeba spp. in two samples (1.7 %).

**DISCUSSION**

Our study indicates a significant number of cases of AGE with infectious aetiology (80 %) during the 4 month study period. Viral, bacterial and parasitic pathogens accounted for 83.7 (77/92), 3.3 (3/92) and 3.3 % (3/92), alone, and 9.8 % (9/92), as co-infections, of all gastroenteritis hospitalizations among children less than 3 years of age. An incidence rate of infectious diarrhea ranging between 39 and 86 % has been reported in several countries in Europe and the rest of the world (Tam et al., 2012; Levidiotou et al., 2009; Colomba et al., 2006; Olesen et al., 2005; Boga et al., 2004; Youssef et al., 2000; Sethi et al., 1989), which is in accordance with our results. However, most of the studies published were focused on viral infections alone (Shojah et al., 2014; Sánchez-Fauquier et al., 2011; Jakab et al., 2009; Fabiana et al., 2007; Oh et al., 2003; Chikhi-Brachet et al., 2002), where virus-positive rates varied from 39 % in France to 62 % in Turkey (Chikhi-Brachet et al., 2002; Akhter et al., 2014). The difference in the viral prevalence seen in the literature might reflect the discrepancies of the studies conducted based on geographical [number of hospital(s) included, region(s) and general population covered], temporal [year(s)/season(s) investigated] or population features (sample size, age of the patients, in/outpatient or hospitalized cases, sporadic alone or mixed
sporadic and outbreak cases), as well as the testing methods used and number of virus targets included for detection. The high burden of infectious diarrhoea cases in our study might be due to the improved clinical assessment of AGE cases (consent for acute infectious intestinal infections of the Bulgarian Society of Infectious Diseases/2009, http://www.bsid-bg.org/index.php/consensi/acute-int-infections) and the use of diagnostic methods with better diagnostic efficacy such as RT-PCR for NoV, AstV and SaV screening.

On the other hand, 20% of the samples remained negative in all diagnostic tests performed. Although the diagnostic tests used in our study are highly sensitive and specific, the proportion of false-negative cases with infectious origin is unknown because of some limitations of our investigation. It must be noted that detection of other diarrhoeal-related pathogens such as picornaviruses or Clostridium difficile, which affect mainly children less than 5 years of age, was not performed. Also, it could not be ruled out that a few specimens with viral aetiology might have been counted as false-negative in the molecular methods used because of the limited sensitivity of this type of diagnostic in samples with low viral load or with presence of strain variants. The use of molecular methods for the detection of bacterial pathogens in recent years has also resulted in increased sensitivity of detection, particularly for fastidious bacterial pathogens such as Campylobacter (Tam et al., 2012).

Our study has shown that viral AGE was characterized by watery stools without mucus/blood and shorter duration of diarrhoea. In contrast, bloody/mucoid stools, abdominal pain and higher temperature were common features in patients with bacterial disease. Dehydration and upper respiratory tract infections were described more often among virus-infected children than in those with bacterial illness. Bacterial disease caused by Salmonella spp. was more severe and characterized by prolonged duration (8–12 days), bloody stools, abdominal cramps and high fever (39–40°C).

Our findings clearly demonstrated that viral infections were the predominant cause of severe diarrhoea requiring hospital admission in summer months. Overall, viruses were found as a sole pathogen in 53% of the samples tested, while bacteria and parasites were detected in 13% in both single and mixed infections. Data from our neighbouring countries Turkey, Greece and Albania (Akhtar et al., 2014; Levidiotou et al., 2009; Fabiana et al., 2007) and also from Hungary, Spain and France (Jakab et al., 2009; Boga et al., 2004; Chikhi-Brachet et al., 2002) are concordant with our findings. Most data for Bulgaria are limited to the aetiology of bacterial and parasitic gastroenteritis (Parmakova et al., 2012, 2013; http://www.ncipd.org/epidemiologicalbulletin). Parmakova et al. (2012) described the aetiology of bacterial AGE in the country for all age groups for the period

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**Fig. 1.** Phylogenetic tree of NoV GI sequences reconstructed using the partial N-terminal capsid region. Bulgarian GI NoVs are marked with ■. The Lordsdale virus with GI.4 genotype was used as outgroup strain.
2007–2011, and showed that prevalence varied from year to year, with predominance of salmonellosis (12–21%), shigellosis (9–11%) and E. coli illnesses (7–11%), and marked seasonality in the summer and autumn (May to October) could be seen. The low incidence of bacterial and protozoal infections observed in our study is in agreement with data from many European countries, such as Greece (Levidiotou et al., 2009), Italy (Colomba et al., 2006), Spain (Boga et al., 2004) and Denmark (Olesen et al., 2005), and clearly demonstrates the successful implementation of Europe-wide control strategies in livestock production and the food industry.

Our investigation has shown that RVs were responsible for the majority of the viral gastroenteritis (62% from all mono-infections; 38/61), but NoV, AstV, enteric AdV and SaV also contributed to diarrhoea-associated morbidity (38%). RV surveillance in Bulgaria started in 2005 and since then it has allowed us to evaluate the incidence of RV infections and the prevalence of circulating genotypes. In our previous investigation of RV diarrhoea cases in children and adults, which covered a period from 2005 to 2008, an average detection rate of 32.4% (27–43%) has been established (Mladenova et al., 2010).

Many countries in the world have reported that RVs are a leading cause of paediatric AGE, accounting for 27–51% of all diarrhoea cases in children under 5 years of age (Parashar et al., 2006; Soriano-Gabarro et al., 2006). The RV prevalence observed in this study is higher than previously found in Bulgaria and in many other studies globally. However, our study only included children less than 3 years of age, in whom the first and usually most severe RV infections requiring hospital admission occur. Thus, the introduction of an RV vaccine in the national immunization programme could decrease significantly the RV morbidity in Bulgaria.

Fig. 2. Phylogenetic tree of NoV GII.4 and GII.3 sequences reconstructed using the partial N-terminal capsid region. Bulgarian GII NoVs are marked with ■.
The present study was designed to investigate the role of viral agents such as NoV, SaV, AstV, AdV and PBV in childhood diarrhoea and their epidemiology features. In the only previous investigation in Bulgaria, our research group has reported 46.8% (220/470) diarrhoea cases with viral aetiology among hospitalized children aged 25 days–12 years in a retrospective 5 month (December 2006–April 2007) survey (Mladenova et al., 2008). In that study, NoVs were found in 12.1% of cases, and a great diversity of genotypes (GII.3, GII.4/2002, GII.4/2006a, GII.4/2006b, GII.9 and GII.Karachi) was observed. In the present study, NoVs were the second major viral agent of acute infantile diarrhoea registered, and genotype diversity was detected again, along with the first detection of NoV GI genotype in Bulgaria. Both GI NoV strains identified belonged to different lineages in the GI.4 cluster, which might indicate a different source of infection. In addition, the globally spread NoV GI.4 variant DenHaag2006b was also detected among five patients who were residents of different locations in Sofia city or Sofia region and were admitted to hospital because of severe gastroenteritis symptoms. All these Bulgarian GI.4/DenHaag2006b strains were identical in sequence analysis, which strongly suggests an outbreak(s) with a common but unrecognized source of infection.

A single possible recombinant, NoV BG638/2009, with GI.b polymerase and GI.3 capsid genome fragments, was also identified. Detection of NoVs that have recombinant characteristics has been recently reported (Giammanco et al., 2012; Rimoldi et al., 2011). These data confirm that recombination is an important genetic mechanism for survival and continuous evolution of viruses. Moreover, additional genetic mechanisms such as point mutations can generate great strain diversity among NoVs. These genetic mechanisms contribute to the emergence of new strains or variants with increased stability in the environment and fitness, and could be a potential obstacle for diagnostics of viral infections, as well as have an influence on the phylogenetic classification and future vaccine designs.

**Fig. 3.** Phylogenetic tree of SaV sequences reconstructed with the partial RdRp-coding region. The Bulgarian SaV is marked with ■. Genotypes according to the capsid-coding sequences are noted with square brackets, while the GI cluster involving SaV strains based on the sequencing of the RdRp-coding region are depicted with a round bracket. Porcine GIII SaV is used as an outgroup strain.
SaVs were detected only in 0.9% of samples included in this study. This detection rate is similar to other reports from Europe where the detection level varies between 0.6% in Italy and 2.0–2.4% in the UK and The Netherlands (Rimoldi et al., 2011; Amar et al., 2007; de Wit et al., 2001). The first detection of a SaV strain in Bulgaria revealed that its polymerase-coding region sequence was closely related to SaVs clustering in the GII genogroup and also to intergenogroup recombinant strains with the GII RdRp/GIV-capsid region. However, if the Bulgarian strain is a possible recombinant or a typical GII SaV, further investigations of its capsid-coding genome fragment are needed.

AstVs were detected in 8.7% (8/92), which is in accordance with our previous data for lower frequency of these infections in hospitalized children (0.2%; Mladenova et al., 2008). Genotyping of AstV strains by sequence analysis was carried out for the first time in Bulgaria, and it revealed the existence of a diverse AstV population. Four of the eight Bulgarian AstVs that were identified as genotype 1a, BG668/2009, BG710/2009, BG741/2009 and BG747/2009 shared high nt and aa inter-strain identity and likely represent a single AstV strain and source of infection. These four isolates together with the isolate BG703/2009, which belongs to genotype 1d, represent classical AstVs with ORF1a and ORF2 of the same type. In contrast, the other two AstV isolates, BG656/2009 and BG704/2009, each detected in a single sporadic gastroenteritis case, are possible recombinants as the ORF1a and ORF2 parts of the genes belonged to different phylogenetic clusters. In a recent paper by Martella et al. (2014), a great lineage diversification in ORF2 due to point mutations, recombination and rearrangements events has been observed, but genetic analysis of ORF1 and ORF2 in parallel will better clarify the AstV diversity.

PBVs were not detected in our investigation. These viruses were first detected in 1988 in stool samples of children and rats (Pereira et al., 1988), and since then have been recognized as a putative infectious agent or opportunistic pathogen in children and adults with/without diarrhoea, immunocompromised persons and kidney-transplant

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**Fig. 4.** Phylogenetic tree of AstV sequences reconstructed with the RdRp-coding (a) and partial capsid-coding (b) regions. Bulgarian AstVs are marked with ■. Genotypes according to the capsid-coding region are noted at the end of the isolate names.
patients (Ganesh et al., 2012), as well as in various animal species with or without enteric symptoms. Nevertheless, PBVs are still rarely detected in the world, and their prevalence ranges from 0.09 % in Argentina and 0.43 % in Italy to 2.1 % in India (Giordano et al., 2008; Cascio et al., 1996; Ganesh et al., 2010).

High prevalence of viral infections, especially with RVs, during the summer months covered by our investigation was an interesting observation. It is in line with the increased circulation of RVs during late summer months in Bulgaria, registered since 2007 (Mladenova et al., 2010; http://www.ncipd.org/epidemiologicalbulletin). Although rarely reported, similar RV peaks during summer have been registered in Spain (Boga et al., 2004) and Taiwan (Chiu et al., 2000). In general, RVs and NoVs cause diarrhoeal illnesses that occur year round, but predominantly during the winter months in countries with temperate climates. Temperature and relatively humid weather conditions have been proposed to contribute to increased RV and NoV infections. However, a few of the virus characteristics such as environmental stability, heat resistance and easy transmission by the faecal–oral route foster the spread of infections also during summer season. Diarrhoeal viruses such as RV, NoV and AstV spread mainly through contact with infected persons, contaminated environmental surfaces or via ingestion of contaminated food or water. Crowding of people and increased person-to-person contact during the summer, increased consumption of fresh fruit, raw vegetables, ice
cream and sea food (mainly bivalves), attendance at recreational water sources or emergence of a new virus variant in the susceptible child population may potentially be factors that favour the spread of virus gastroenteritis agents during the summer months. However, to date, we do not have a definitive explanation for the seasonality of viral infections or for the differences seen in seasonal patterns between different parts of the world.

CONCLUSION

In summary, our study is, we believe, the first systematic investigation of the aetiology spectrum of AGE in a small cohort of children under 3 years of age in Bulgaria, covering detection of 14 diarrhoeal infectious pathogens, the most common viral (six), bacterial (five) and parasitic (three) agents. Infectious origin was established in 80% of the samples tested. Of them, viruses were detected in 75% of the positive samples, which confirms their leading role as causative agents of acute childhood diarrhoea. Our study also revealed high morbidity associated with viral infections during summer months and a significant genetic diversity amongst these pathogens, ruling out that the high prevalence of RV or NoV was due to outbreaks. Overall, 20% of the stool samples investigated for viral, bacterial and parasitic pathogens remained negative in all diagnostic tests performed. Widening of routine laboratory diagnostics toward other bacterial or viral (Clostridium difficile, entero/parechoviruses) or new emerging viral agents (Aichi virus, bocavirus, torovirus) and the inclusion of molecular methods for the investigation of bacterial pathogens, in particular those that are challenging to culture, will help improve AGE management in health care settings in Bulgaria.

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