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Distribution of rotavirus genotypes in three Croatian regions among children ≤5 years of age (2012–2014)

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ABSTRACT

Objectives: Rotavirus is the major cause of severe diarrhea in young children worldwide. In countries like Croatia, where rotavirus vaccine has not been introduced in the national immunization program, prospective surveillance is necessary to establish the diversity of rotavirus strains. The aim of this study was to describe the prevalence and geographical distribution of rotavirus strains in Croatia and to detect the possible emergence of novel strains.

Methods: The study was conducted among children ≤5 years of age with acute gastroenteritis at three hospitals located in different geographical regions of Croatia, during the years 2012 to 2014. Rotavirus was detected in stools using an immunochromatographic assay and then sent for further molecular analysis.

Results: Genotyping of 822 rotaviruses showed that the predominant circulating strain was G1P[8] (61.9%), followed by G2P[4] (19.5%), G1P[4] (3.9%), and G3P[8] (2.9%). A high prevalence of reassortants among common human rotavirus genotypes was detected (7.7%). Possible zoonotic reassortants were found, including G8 and G6 strains. The latter is described for the first time in Croatia.

Conclusions: This study represents pre-vaccination data that are important for decisions regarding immunization strategies in Croatia. The high prevalence of 'common' rotavirus strains circulating in Croatia may advocate for rotavirus vaccine introduction, but further surveillance is necessary to monitor the possible emergence of novel genotypes.

Introduction

Diarrhea is one of the leading causes of mortality among young children and is responsible for almost 500 000 deaths annually worldwide (Wang et al., 2016; Troeger et al., 2017). Despite the decreasing number of cases, rotavirus (RV) is still the main global cause of diarrhea-associated morbidity and mortality in children younger than 5 years of age; it is considered responsible for nearly 260 million diarrhea episodes and one-third of all diarrhea-related deaths in 2016 (Troeger et al., 2018). In developed countries, the mortality rates associated with RV are significantly lower than those in developing countries (Tate et al., 2016; Troeger et al., 2018), but RV infection is still responsible for considerable morbidity and health costs (Rheingans et al., 2006; Troeger et al., 2018).

Group A RVs are classified into G and P types based on the sequence diversity of the genes encoding the outer viral proteins VP7 (glycoprotein) and VP4 (protease-sensitive protein)

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(Matthijnssens et al., 2008). Comprehensive reviews of G and P type prevalence data before the introduction of RV vaccines have indicated the global importance of five genotypes: G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8]; however, a great diversity of G/P combinations has also been identified (Gentsch et al., 2005; Santos and Hoshino 2005; Bányai et al., 2012). Unusual and novel antigen combinations have continued to emerge, some of them spreading globally, such as G12P[8] (Matthijnssens et al., 2010), while some strains have become important regionally or locally, such as G8P[6] in parts of Africa and G12P[6] in parts of Asia (Cunliffe et al., 2010; Ansari et al., 2013). Genetic interaction by reassortment among co-circulating RVs contributes significantly to the great diversity of G/P combinations (Iturriza-Gómara et al., 2001). Also, RVs are ubiquitous in the animal kingdom, and interspecies transmission and exchange of genetic material between the animal and human strains is not rare, resulting in the emergence of novel RV strains, some with epidemiological significance (Iturriza-Gómara et al., 2004). The distribution of RV G/P combinations shows geographical and temporal fluctuations. Not only can the dominance of a certain genotype change dramatically from year to year and from country to country, but the incidence of RV genotypes in different regions within the same country can differ during the same year (Santos and Hoshino 2005; Ogilvie et al., 2011; Bányai et al., 2012).

In response to the substantial burden of RV disease among children worldwide, RV vaccines have been developed as the most effective preventive strategy. Two live-attenuated oral RV vaccines, RotaTeq (Merck, Whitehouse Station, NJ, USA), a pentavalent (G1, G2, G3, G4, P[8]) bovine–human reassortant vaccine, and Rotarix (GSK Biologicals, Rixensart, Belgium), a monovalent (G1P[8]) human strain vaccine, are currently recommended by the World Health Organization (WHO) for introduction into routine immunization programs worldwide (WER, 2009). Recently, two vaccines have been added to the WHO list of prequalified vaccines: Rotavac (Bharat Biotech International Ltd, Hyderabad, India), which contains a G9P [11] human strain, and Rotasil (Serum Institute of India Ltd, Pune, India), a pentavalent (G1, G2, G3, G4, G9) bovine–human reassortant vaccine (WHO, 2019). In Croatia, RV vaccines are not included as part of the national immunization program and are only recommended for children at higher risk (Hrvatski zavod za javno zdravstvo, 2017). Before the introduction of an RV vaccine into a national immunization program, it is important to obtain local epidemiological data on the burden of RV disease, including the age distribution, seasonal trends, and assessment of the circulating genotypes, in order to create effective strategies to control the disease (Vesikari et al., 2008; Matthijnssens et al., 2009).

In Croatia, data on the molecular epidemiology of the RV strains responsible for acute gastroenteritis are limited. One study in 2007 described the genotype distribution of RVs in Central Croatia (Zagreb metropolitan area) (Tcheremenskaia et al., 2007), but no studies have included other regions of the country.

The aim of this study was to assess the prevalence of RV genotypes in three different regions of Croatia (North, Central, and South) in order to determine possible differences in geographical distribution of RV genotypes and monitor the possible emergence of novel RV strains in the selected regions. The data can be used to direct policy decisions with regard to the introduction of RV vaccine into the national immunization program.

Materials and methods

This prospective observational study was conducted during two consecutive RV seasons, from July 2012 to July 2014, at three hospitals representing three geographical regions of Croatia (Figure 1): County Hospital Čakovec (situated in the northernmost Croatian county), University Hospital for Infectious Diseases "Dr Fran Mihaljević", Zagreb (city of Zagreb and Zagreb County – Central Croatia), and University Hospital of Split (city of Split and Split County – South Croatia). The study was approved by the ethics committees of all three institutions and informed consent was obtained from the patients' caregivers before inclusion.

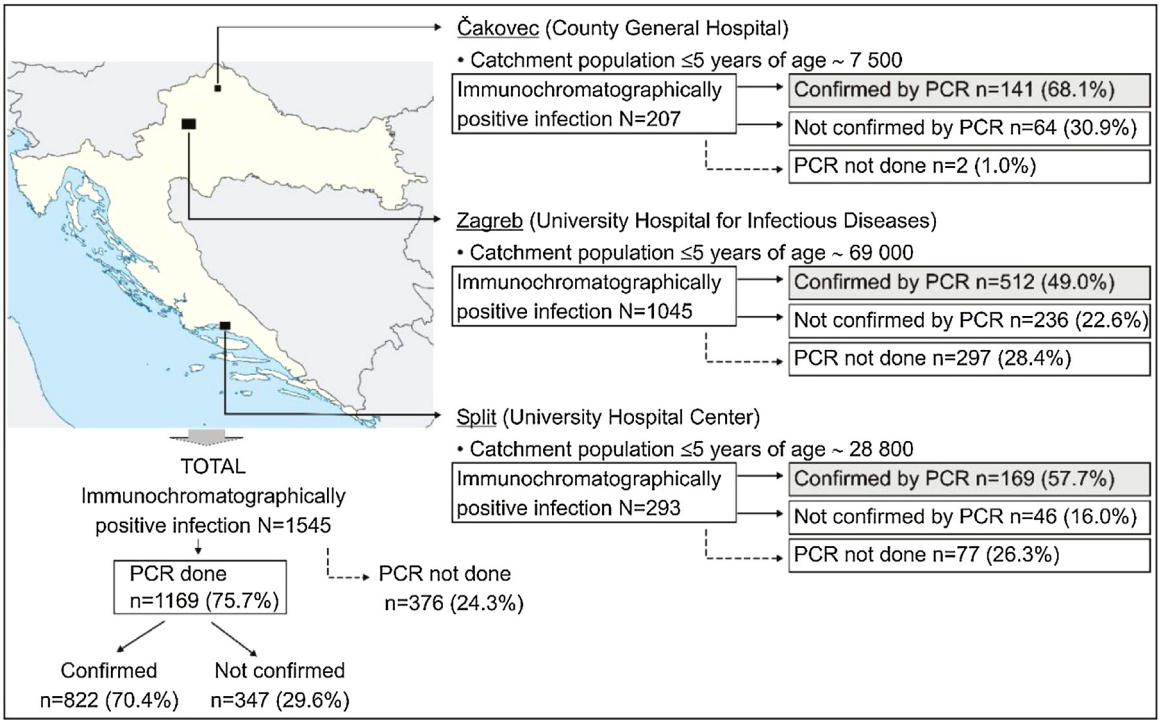

Figure 1. Schematic representation of the geographical locations of the centers included in the study. Data on the catchment population ≤5 years of age are based on the 2011 census data (available at: https://www.dzs.hr/Hrv/censuses/census2011/results/censustabsxls.htm, accessed September 15, 2018).

Diagnosis of rotavirus infection and patient management

All children aged ≤5 years with a diagnosis of acute gastroenteritis, hospitalized or treated through the emergency departments during the observation period were included in the study. Acute gastroenteritis was defined as diarrhea (≥3 looser-than-normal stools per day), with or without vomiting (≥1 episode of forceful emptying of partially digested stomach contents ≥1 hour after feeding per day). Children with community-acquired and hospital infections (patients hospitalized for other reasons who developed acute gastroenteritis at >48 h after admission, or patients who developed the disease within 72 h after hospital discharge) were included. Children living in children's homes and those with chronic diarrhea, inflammatory bowel diseases, or immune deficiencies were not included. Epidemiological and demographic data (age, sex, geographical location, date of onset of disease, date of sample collection) were collected and entered into a database for linkage to the genotyping data.

One stool sample per patient was obtained and tested for RV and the presence of adenoviral antigen using a single commercial immunochromatographic assay, Rota-AdenoGnost test (BioGnost, Hannover, Germany). RV-positive samples were stored at −20 °C at the local microbiology laboratories until shipment for molecular typing at the Department of Clinical Microbiology, University Hospital for Infectious Diseases, Zagreb, Croatia. Viral RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. A reverse transcriptase PCR (RT-PCR) was conducted using the Qiagen OneStep RT-PCR Kit (Qiagen, Hilden, Germany) with primers VP7-F and VP7-R (Gómara et al., 2001) and VP4-F and VP4-R (Gentsch et al., 1992), using a Veriti thermal cycler (Applied Biosystems, Waltham, MA, USA). PCR typing was performed according to the protocol of the European Rotavirus Detection and Typing Methods (available at the EuroRotaNet website http://www.eurorota.net). Samples that were non-typeable by PCR were submitted for DNA sequencing using primers VP7-F, VP7-R, VP4-F, and VP4-R. The PCR amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing was performed at Macrogen Europe (Amsterdam, The Netherlands). Genotypes were assigned using the RotaC v1.1 genotyping tool (http://rotac.regatools.be).

Data analysis

Patient characteristics and observed crude prevalence rates were summarized by site (region), age, and sex. The intended stratification by age (≤12 months, 12–36 months, and >36 to 60 months) was changed to ≤12 and >12 months, since only 9.7% of those for whom PCR was done and only 8.7% of those with PCR-confirmed RV infection were older than 36 months. Prevalence rates by site or age or sex adjusted for the other two factors were obtained from generalized linear models (by inverse link function). Differences of interest were expressed as the relative difference (adjusted prevalence ratios (PR), by modified Poisson regression with robust error variance (models risk instead of odds) (Zou, 2004)), or as an absolute difference between adjusted prevalence rates (by the method of variance estimates recovery (Newcombe, 2016)). SAS 9.4 for Windows was used for the analysis (SAS Institute Inc., Cary, NC, USA).

Results

A total of 1545 children with an immunochromatographically positive RV infection were enrolled. The centers contributed patients in proportion with their catchment population (Figure 1). Due to technical reasons, genotyping was not performed in 376 (24.3%) cases (Figure 1). RV infection was confirmed by RT-PCR in 822/1169 (70.4%) processed samples (Figure 1). This proportion was higher in Split (South Croatia) than at the other two sites (Figure 1, Table 1). It was also higher in children ≤12 months of age compared to those >12 months of age (Table 1); however, children included in Split were considerably younger than children included at the two other centers (71.8% were ≤12 months of age vs. approximately 25% in the other centers) (Table 1). Adjusted differences between centers and between age groups were minor (Table 2).

In the 822 children with PCR-verified infection, G1 was by far the predominant G-type, followed by G2 (three-fold lower prevalence), while G3, G9, and G4 were sporadic (~3%) (Figure 2). Only three cases of 'uncommon' G-types (G6, G8) were observed (Figure 2). Of the P-types, P[8] by far prevailed, followed by P[4] (three-fold less) and one case of P[6] ('uncommon' type) (Figure 2). A number of different G/P combinations were observed (Table 3). Overall, G1P[8]

Table 2
Adjusted prevalence of PCR-confirmed rotavirus infections by site, age group, and sex; differences are expressed as (adjusted) prevalence ratios (PR).

	Prevalence (%) (95% CI)	PR (95% CI)	*p*-Value
Site			
Zagreb[a]	69.9 (66.4–73.5)	1.00 (reference)	–
Split[b]	77.3 (71.7–83.3)	1.11 (1.01–1.22)	0.039
Čakovec[c]	70.3 (64.1–77.1)	0.99 (0.90–1.10)	0.909
Age			
≤12 months	75.6 (71.4–80.2)	1.00 (reference)	–
>12 months	69.3 (65.3–73.4)	0.92 (0.84–0.99)	0.036
Sex			
Female	74.2 (70.1–78.6)	1.00 (reference)	–
Male	70.6 (66.9–74.5)	0.95 (0.88–1.02)	0.194

PCR, polymerase chain reaction; CI, confidence interval. The probability of a PCR-confirmed infection was analyzed in a modified Poisson regression model with robust error variance (models ln[risk] instead of ln[odds]).
[a] Central Croatia.
[b] South Croatia.
[c] North Croatia.

Table 1
Subject characteristics; data are count (%) or median (range).

	All	By site (region)			By age (months)		By sex	
		Zagreb[a]	Split[b]	Čakovec[c]	≤12	>12	Male	Female
ICT (+) and PCR done	1169	748	216	205	417	752	653	516
Age (months)	14.4 (0.03–60)	17.5 (0.13–60)	4.40 (0.03–58)	13.2 (0.30–56)	–	–	13.4 (0.03–60)	15.2 (0.26–58)
Age 0–12 months	417 (35.7)	209 (27.9)	155 (71.8)	53 (25.8)	–	–	254 (38.9)	163 (31.6)
Age >12 months	752 (64.3)	539 (72.1)	61 (28.2)	152 (74.2)	–	–	399 (61.1)	353 (68.4)
Male	653 (55.9)	403 (53.9)	142 (65.7)	108 (52.7)	254 (60.9)	399 (53.1)	–	–
Confirmed by PCR	822 (70.4)	512 (68.4)	169 (78.2)	141 (68.8)	315 (75.5)	507 (67.4)	452 (69.2)	370 (71.7)

ICT (+), immunochromatographically positive; PCR, polymerase chain reaction.
[a] Central Croatia.
[b] South Croatia.
[c] North Croatia.

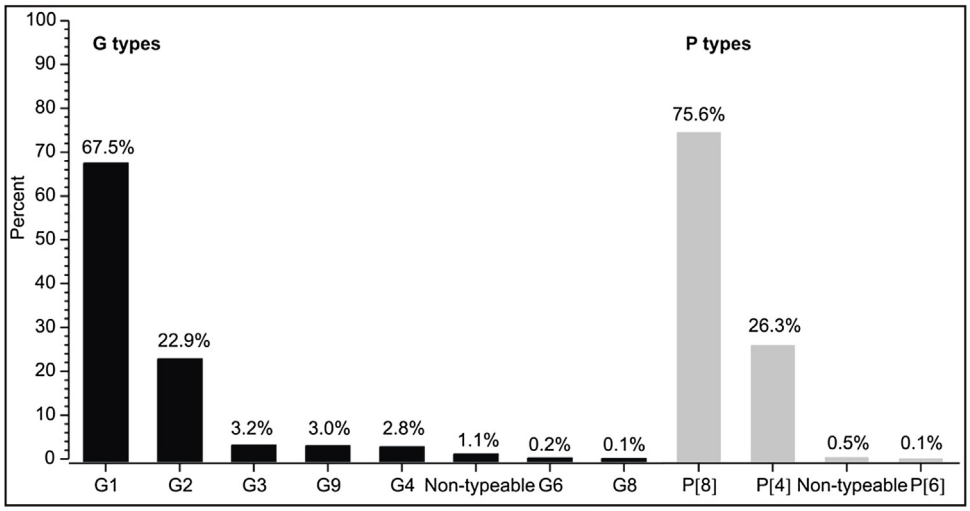

Figure 2. Overall prevalence of individual G and P types in 822 children with PCR-confirmed rotavirus gastroenteritis.

with 61.9% and G2P[4] with 19.5% accounted for 81.4% of all genotypes (Table 3). Other genotypes were identified in <5% of the samples, and G9P[8] accounted for 2.3% of the samples (Table 3). Mixed infections with different P- or G-types were identified in 1.62% of cases. Strains that were non-typeable for the G- or P-type accounted for 1.62 % of the samples (Table 3). The prevalence of the two most common genotypes (G1P[8], G2P[4]) appeared different across regions (Table 3), with slight differences also between age groups and by sex (Table 3). The age and sex-adjusted prevalence (Table 4) of G1P[8] was approximately 20% higher, and the prevalence of G2P[4] was approximately 20% lower in the North compared to the Central and South Croatia regions. Observed prevalence rates of the less common genotypes also varied somewhat by region, age, and sex (Table 3). Age and sex-adjusted prevalence rates (Table 4) indicated the following: (1) a moderate prevalence of G1P[4] (around 10%) in the North compared to <2.2% in other regions; (2) a notable prevalence of P[8] (non-typeable G) in the North (around 4.4%) compared to sporadic (<0.5%) in other

Table 3
Characteristics and identified rotavirus genotypes in children with PCR-confirmed infection; data are count (%) or median (range).

	All	By site (region)			By age (months)		By sex	
		Zagreb[a]	Split[b]	Čakovec[c]	≤12	>12	Male	Female
Number	822	512	169	141	315	507	452	370
Age (months)	13.5 (0.03–60)	17.6 (0.13–60)	4.00 (0.03–54)	13.2 (0.30–56)	–	–	13.2 (0.03–60)	14.8 (0.26–57)
Age 0–12 months	315 (38.3)	143 (27.9)	131 (77.5)	41 (29.1)	–	–	185 (40.9)	130 (35.1)
Age >12 months	507 (61.7)	369 (72.1)	38 (22.5)	100 (70.9)	–	–	267 (59.1)	240 (64.9)
Male	452 (55.0)	270 (52.7)	112 (66.3)	70 (49.7)	185 (58.7)	267 (52.7)	–	–
G1P[8]	509 (61.9)	293 (57.2)	108 (63.9)	108 (76.6)	213 (67.6)	296 (64.7)	291 (64.4)	218 (58.9)
G2P[4]	160 (19.5)	129 (25.2)	27 (16.0)	4 (2.8)	49 (15.6)	111 (21.9)	78 (17.3)	82 (22.2)
G1P[4]	32 (3.9)	13 (2.5)	3 (1.8)	16 (11.3)	8 (2.5)	24 (4.7)	18 (4.0)	14 (3.8)
G3P[8]	24 (2.9)	6 (1.2)	17 (10.1)	1 (0.7)	14 (4.4)	10 (2.0)	14 (3.1)	10 (2.7)
G2P[8]	22 (2.7)	22 (4.3)	0	0	5 (1.6)	17 (3.3)	12 (2.6)	10 (2.7)
G9P[8]	19 (2.3)	14 (2.7)	5 (2.9)	0	6 (1.9)	13 (2.6)	10 (2.2)	9 (2.4)
G4P[8]	18 (2.2)	13 (2.5)	4 (2.4)	1 (0.7)	5 (1.6)	13 (2.6)	12 (2.6)	6 (1.6)
GNT P[8]	8 (1.0)	1 (0.2)	1 (0.6)	6 (4.3)	4 (1.3)	4 (0.8)	3 (0.7)	5 (1.3)
G9P[4]	5 (0.6)	5 (1.0)	0	0	2 (0.6)	3 (0.6)	4 (0.9)	1 (0.3)
G4P[4]	4 (0.5)	4 (0.8)	0	0	1 (0.3)	3 (0.6)	1 (0.2)	3 (0.8)
G1P[4]+P[8]	4 (0.5)	1 (0.2)	0	3 (2.1)	0	4 (0.8)	1 (0.2)	3 (0.8)
G1P[NT]	3 (0.4)	2 (0.4)	0	1 (0.7)	2 (0.6)	1 (0.2)	2 (0.4)	1 (0.3)
G1+G2P[4]+P[8]	3 (0.4)	3 (0.6)	0	0	0	3 (0.6)	0	3 (0.8)
G1+G2P[4]	1 (0.12)	1 (0.2)	0	0	1 (0.3)	0	0	1 (0.3)
G1+G3P[4]+P[8]	1 (0.12)	0	1 (0.6)	0	1 (0.3)	0	1 (0.2)	0
G1+G3P[8]	1 (0.12)	0	1 (0.6)	0	1 (0.3)	0	1 (0.2)	0
G1+G9P[8]	1 (0.12)	0	1 (0.6)	0	1 (0.3)	0	0	1 (0.3)
G2P[NT]	1 (0.12)	1 (0.2)	0	0	1 (0.3)	0	1 (0.2)	0
G2P[4]+P[8]	1 (0.12)	1 (0.2)	0	0	0	1 (0.2)	0	1 (0.3)
G4P[6]	1 (0.12)	1 (0.2)	0	0	0	1 (0.2)	1 (0.2)	0
G6P[4]	1 (0.12)	1 (0.2)	0	0	0	1 (0.2)	0	1 (0.3)
G6P[4]+P[8]	1 (0.12)	0	1 (0.6)	0	0	1 (0.2)	1 (0.2)	0
G8P[8]	1 (0.12)	1 (0.2)	0	0	0	1 (0.2)	0	1 (0.3)
GNT P[4]	1 (0.12)	0	0	1 (0.7)	1 (0.3)	0	0	1 (0.3)

PCR, polymerase chain reaction; NT, non-typeable.
[a] Central Croatia.
[b] South Croatia.
[c] North Croatia.

Table 4
Age and sex-adjusted prevalence (%) (95% CI) of individual genotypes by site (region). Only genotypes with an observed prevalence of ≥1.0% at any site are shown. Values in bold are those that numerically stand out at a particular site vs. Zagreb (Central Croatia) as the 'reference' site. The column 'Difference' contains the absolute prevalence difference: value in bold – 'reference'.

Genotype	Zagreb[a]	Split[b]	Čakovec[c]	Difference (95% CI)
G1P[8]	59.5 (54.8–64.1)	60.3 (52.2–67.9)	**78.4 (70.9–84.4)**	18.9 (10.1, 26.5)
G2P[4]	23.6 (19.7–27.9)	17.9 (12.5–25.1)	**2.6 (0.9–6.7)**	−21.0 (−25.6, −15.3)
G1P[4]	2.2 (1.2–4.1)	1.9 (0.6–5.9)	**10.2 (6.0–16.9)**	8.0 (3.4, 14.8)
G3P[8]	1.1 (0.5–2.5)	**11.0 (6.5–17.8)**	0.7 (0.1–4.6)	9.9 (5.2, 16.7)
G2P[8]	4.0 (2.5–6.4)	0	0	–
G9P[8]	2.4 (1.2–4.4)	3.4 (1.4–8.1)	0	–
G4P[8]	2.0 (1.0–4.0)	2.4 (0.9–6.6)	0.6 (0.1–4.1)	–
GNT P[8]	0.2 (0.0–1.5)	0.5 (0.0–3.8)	**4.4 (1.9–9.9)**	4.2 (1.4, 9.7)
G9P[4]	0.8 (0.3–2.6)	0	0	–
G1P[4]+P[8]	0.2 (0.0–1.3)	0	1.8 (0.5–6.3)	–

CI, confidence interval; NT, non-typeable. A separate generalized linear model was fitted to probability of individual genotypes.
[a] Central Croatia.
[b] South Croatia.
[c] North Croatia.

regions; (3) a moderate prevalence of G3P[8] (around 11%) in the South compared to sporadic (≤1.0%) in the other regions.

Discussion

This is the first Croatian national study to describe the molecular epidemiology of RV strains among children under 5 years of age in three different regions of Croatia: North, Central, and South. One previous study conducted in 2005–2006 in a wider area of Central-Eastern Europe (Croatia, Czech Republic, Slovenia, Albania, and Bulgaria) included RV samples only from the Zagreb metropolitan area, i.e. Central Croatia (Tcheremenskaia et al., 2007).

RV gastroenteritis associated with severe dehydration mainly affects children under 5 years of age. However, in developed countries, RV infections are more common in children 9–15 months of age (Zeller et al., 2010), while in the low-income countries, most of the RV infections are detected in children <1 year of age (Gasparinho et al., 2017; Boni-Cisse et al., 2018). The age distribution of patients overall and in Central and North Croatia showed a pattern similar to that in developed countries (median age 14.4, 17.5, and 13.2 months, respectively). Patients in South Croatia tended to be much younger, with a median age of 4.4 months.

Five RV strains ('common' genotypes G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8]) were found to be responsible for the majority of severe RV infections worldwide in the pre-vaccine era (Gentsch et al., 2005; Santos and Hoshino, 2005; Bányai et al., 2012) and have remained predominant in the post-vaccine licensure period (Dóró et al., 2014). We detected numerous G- and P-types of RV circulating in the Croatian population: seven G-types (G1, G2, G3, G4, G6, G8, G9) and three P-types (P[8], P[4], P[6]) occurring in diverse genotype combinations. However, the majority of cases in all three regions were caused by genotype G1P[8] (57.2% in Central, 63.9% in South, and 76.6% in North, respectively). The prevalence of the globally 'common' G/P combinations was 88.8% overall and between 81% and 95% across the regions. This pattern of high prevalence of 'common' RV strains is typical of the developed countries of North America, Europe, and Australia (Santos and Hoshino, 2005) and is in contrast with previous findings in Croatia, where the G1P[8] genotype was detected in less than 22% of the samples and the total prevalence of 'common' G/P combinations was less than 60% (Tcheremenskaia et al., 2007). This suggests that the distribution and prevalence of particular RV genotypes changes over time and that one genotype can be replaced by another, as described in several studies (Ogilvie et al., 2011; Bányai et al., 2012; Dóró et al., 2014).

Interestingly, the G9 strain, which has emerged as the one of the globally most common G-types, including in Europe (Cubitt et al., 2000; Martella et al., 2003; Santos and Hoshino, 2005; Van Damme et al., 2007; De Rougemont et al., 2009, 2011; Bányai et al., 2012), was found in only 25 samples (3.02%) in the present study: as G9P[8] in 2.3%, G9P[4] in 0.6%, and as a part of a mixed genotype in 0.12% of the samples. A similar unusually low rate of G9 has been reported previously in Croatia, where G9 accounted for only 2.2% of the G-types (Tcheremenskaia et al., 2007). Of note, G9 was not found in North Croatia in the present study.

Besides the 'common' human RV strains, 'uncommon' G/P combinations were also found that might have originated from reassortment between common human strains or animal and human strains (Iturriza-Gómara et al., 2001; Gentsch et al., 2005). Genotypes that presented reassortments among the common human RV genotypes were G1P[4] (3.9%), G2P[8] (2.7%), G9P[4] (0.6%), and G4P[4] (0.5%). Of note, the G1P[4] genotype was the third most frequent genotype overall and the second most common in North Croatia, where it accounted for 11.3% of all genotypes. Possible human–animal hybrid RV strains were detected sporadically (total 0.36%): G4P[6], G6P[4], and G8P[8]. Such a high prevalence of G1P[4] genotype has rarely been reported (Abdel-Haq et al., 2003). Furthermore, this unusually high percentage of 'uncommon' genotypes (8.06%), especially human–human reassortants (7.7%), is not typical for European countries, where it has been reported with a prevalence of around 1% (Iturriza-Gómara et al., 2011). Similar findings have already been described in Croatia and suggest probable horizontal transmission of some reassortant strains, since their prevalence remained high in the previous study (Tcheremenskaia et al., 2007) and in the current study.

Another interesting finding is the appearance of the G6 type, which has not been described previously in Croatia (Tcheremenskaia et al., 2007). The G6 type is one of the most common G types found in cattle (Martella et al., 2010), but has also been identified in children with acute gastroenteritis worldwide, mostly sporadically (Rahman et al., 2003; De Grazia et al., 2011; Iturriza-Gómara et al., 2011; Afrad et al., 2013). In some countries, such as Hungary, the G6 type was detected with a higher prevalence of 1% and became one of the emerging genotypes (Bányai et al., 2004). In the present study, it was found in only two samples: in a combination with P[4] and as a part of a mixed genotype G6P[4]+P[8]. Interestingly, the samples were from two different geographical regions – one from the South and one from the North Croatia region. Since G6 strains are very common in cattle, they were probably transmitted to humans directly from an animal reservoir (De Grazia et al., 2011) or arose by interspecies transmission accompanied by reassortment (Bányai et al., 2003). It will be important to monitor the transmission and prevalence of the G6 type in Croatia, since it is not incorporated in the available RV vaccines and it is not known

whether vaccine strains would ensure sufficient heterotypic protection against this type.

Unlike the previous report of a high prevalence of mixed genotypes in Croatia (Tcheremenskaia et al., 2007), in this study they were observed in less than 2% of the samples, similar to the findings in developed countries (Iturriza-Gómara et al., 2011). Mixed infections are significant because they may contribute to the generation of unusual antigen combinations by gene reassortment in vivo (Bányai et al., 2004), and their prevalence is often higher in developing countries where greater variation of different genotype combinations is also usually detected (Gentsch et al., 2005).

This study revealed significant differences in distribution of the most common genotypes among different regions. It has already been reported that the prevalence of individual RV genotypes shows continental and subcontinental variations, but it can also differ between regions in the same country (Santos and Hoshino, 2005; Ogilvie et al., 2011; Bányai et al., 2012). It is interesting that greater differences were found between North and Central Croatia, which are geographically closely related, than between Central and South Croatia, which are not in a direct geographical contact. It is assumed that many factors affect RV diversity in a certain region: the intensity of the horizontal transmission between humans (which in Croatia is also driven by extensive migration from South to Central Croatia), reassortment of human strains, zoonotic transmission, and probably some other factors.

This study has several limitations. A considerable proportion of immunochromatographically positive RV samples (16.0–30.9%, depending on the center) could not be amplified by RT-PCR. Since immunochromatographic tests for the detection of RV are highly specific (97–100%) (Kaplon et al., 2015), such a high percentage of RV infections not confirmed by PCR cannot be explained only by false-positive results. Probable explanations include technical issues, inadequate storage and transportation of the samples (destruction of viral RNA), or too low viral load that was not sufficient for further analysis. Another limitation is that not all of the immunochromatographically positive samples were sent for further molecular analysis (1.0–28.4%), which could have affected the final results. Finally, the study was conducted in the major hospital centers in Croatia, which may not be representative of the whole population.

In conclusion, this study provides pre-vaccination data important for decisions regarding immunization strategies in Croatia, where RV vaccine has still not been introduced into the national immunization program. The high prevalence of 'common' RV strains circulating in Croatia may advocate for RV vaccine introduction. However, a possible change in the predominant RV strains and emergence of the new reassortants in circulation highlight the need for continuous surveillance.

Conflict of interest

Maja Vrdoljak and Goran Tešović have received personal fees from GlaxoSmithKline Biologicals SA. The remaining authors have no conflicts of interest to report.

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Ethical approval

The study protocol was approved by the ethics committees of all three institutions where the study was conducted.

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