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Molecular characterization of rotavirus strains from pre- and post-vaccination periods in a country with low vaccination coverage: The case of Slovenia

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ABSTRACT

Rotavirus vaccination started in Slovenia in 2007 on a voluntarily basis. The vaccination rate is relatively low (up to 27%) and no increasing trend is observed. We present rotavirus genotype distribution among children hospitalized for rotavirus gastroenteritis in Slovenia. Eight consecutive rotavirus seasons were followed, from 2005/06 to 2012/13, and 113 strains of the most common rotavirus genotypes were randomly selected for molecular characterization of rotavirus VP7 and VP4 (VP8*) genome segments. During the vaccine introduction period, from 2007 to 2013, rotavirus genotype prevalences changed, with G1P[8] decreasing from 74.1% to 8.7% between 2007/08 and 2010/11 seasons, replaced by G4P[8] and G2P[4], with up to 52.0% prevalence. Comparable analysis of VP7 and VP8* genome fragments within G1P[8] genotype lineages revealed considerable differences for rotavirus strains circulating before and during the vaccination period. The G1P[8] rotavirus strains from the pre-vaccination period clustered in a phylogenetic tree within Rotarix®-like VP7 and VP8* lineages. However, since 2007, the majority of G1P[8] strains have shifted to distant genetic lineages with lower nucleotide (88.1–94.0% for VP7 and 86.6–91.1% for VP8*) and amino acid (93.8–95.2% for VP7 and 85.3–94.6% for VP8*) identities to the vaccine Rotarix® strain. This change also resulted in a different deduced amino acid profile at the major VP7 and VP8* antigenic epitopes.

1. Introduction

Group A rotaviruses are the leading cause of acute gastroenteritis in children worldwide (Parashar et al., 2006). Although hygiene is a very important preventive measure against the disease, it does not provide an efficient tool for the termination of virus transmission and lowering the disease burden in society. This is evident from comparing morbidity data for developing and industrialized countries. The incidence of rotavirus disease in industrialized countries without rotavirus vaccination is still high, which contributes significantly to the hospitalization rate and physician visits of young children. High direct and indirect costs related to the disease thus justify the development and use of an effective rotavirus vaccine (Parashar et al., 2006).

In rotavirus pathogenesis, the outer capsid glycoprotein VP7 and the protease sensitive protein VP4 have a specific role in the early steps of the virus replication cycle. They express antigenic properties of rotaviruses and possess specific neutralization epitopes (Trask et al., 2012). Neutralizing antibodies can either physically disable virus attachment to the receptor or stabilize the outer layer, thus inhibiting conformational changes needed for rotavirus entry into the cell and uncoating of the outer capsid layer (Aoki et al., 2009). Rotavirus infection can be terminated during these early steps if specific neutralizing antibodies are present.

The genetic characteristics of VP7 and VP4 rotavirus genes are of significant interest for rotavirus molecular epidemiology, determining the dual genotype classification (Gentsch et al., 1992; Gouvea et al., 1990). Genotypes G (for VP7) and genotypes P (for VP4) are assigned to each rotavirus strain based on nucleotide sequence identities. A similar genotyping protocol has also been proposed for other genome segments, comprising the whole genome classification proposed by the Rotavirus Classification Working Group (RCWG) (Matthijnssens et al., 2011, 2008). To date, 27 G and 37 P genotypes have been described, with more than 60 G–P combinations found, of which only 10 have been detected in humans (Matthijnssens and Van Ranst, 2012; Santos and

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Hoshino, 2005). Globally, there are 5 major rotavirus genotype combinations that are the most prevalent in childhood diarrhea: G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] (Banyai et al., 2012). In the 1990s, G12P[8] appeared and became the sixth most common genotype in some geographical areas (Matthijnssens et al., 2010).

Zoonotic potential and interspecies transmission of group A rotaviruses are high, which was already noted in early research steps of rotavirus epidemiology in the 1980s (Cook et al., 2004). However, only a small proportion of zoonotic transmitted strains cause major outbreaks or spread quickly within the human population. Rather, there are sporadic cases with no major impact on the rotavirus epidemiology (Gentsch et al., 2005; Martella et al., 2010; Papp et al., 2013; Steyer et al., 2008). Nevertheless, following rotavirus epidemiology is of high importance, also for detecting these zoonotic strains, since they could undergo genome reassortment and influence the genotype pattern over a longer time scale, as was shown in the case of G9P[8] (Iturriza-Gomara et al., 2000; Matthijnssens et al., 2010).

Following rotavirus molecular epidemiology has been an especially high priority in the last seven years since two rotavirus vaccines, Rotarix® (GlaxoSmithKline) and RotaTeq® (Merck Sharp & Dohme), were introduced and have been in use (Lopman et al., 2012). Some countries have included this vaccine in the national vaccination program and achieved very good vaccination coverage in infants. In those countries, rotavirus incidence and hospitalization rates for rotavirus gastroenteritis have decreased significantly (Buttery et al., 2011; Dennehy, 2012; Msimang et al., 2013; Payne et al., 2013; Standaert et al., 2013; Vesikari et al., 2013). The effectiveness of the two rotavirus vaccines was confirmed in large prelicensure clinical trials and they showed good protection for the most common rotavirus genotypes (Ruiz-Palacios et al., 2006; Vesikari et al., 2006). In the post-licensure period, rotavirus strain surveillance studies are on-going in order to collect epidemiological data on vaccine influence on rotavirus molecular epidemiology and to ensure that the selective pressure of the vaccine will not result in filtration of vaccine escape mutants (Jain et al., 2014; Leite et al., 2008; Lopman et al., 2012; Zeller et al., 2010).

In our study, archived and current circulating rotavirus strains were selected for comparative molecular analysis of the most common genotype strains in Slovenia during eight consecutive rotavirus seasons (2005/06–2012/13). Phylogenetic clustering and a detailed analysis of amino acid residues at the antigenic epitopes were carried out to explain genotype variation after the vaccine introduction period in Slovenia.

2. Materials and methods

2.1. Sample collection

Stool samples were collected mainly from hospitalized children presenting with acute gastroenteritis. Unfortunately, clinical data were not available for most of the children since this was a retrospective study and archived stool samples were analyzed. Stool samples from confirmed rotavirus infections were collected in regional laboratories from six of eight healthcare regions in Slovenia and sent to the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana. Stool samples were stored at −20 °C prior to genotyping and molecular analysis of rotavirus strains. In the study period of the 8 consecutive rotavirus seasons, from 2005/06 to 2012/13, a total of 3227 rotavirus strains were analyzed.

A 10% stool suspension was prepared in 0.2 M PBS (pH value 7.4) and centrifuged for 5 min at 1600×*g* for clarification. An aliquot of 200 µl supernatant was used further for total nucleic acid extraction with an iPrep™ PureLink® Virus Kit (Life Technologies, Invitrogen Division, Carlsbad, CA). Extracted nucleic acid was stored at −80 °C until further use for genotyping.

2.2. RT-PCR and genotyping

For rotavirus genotyping, VP7 and VP8* segments were initially amplified, using VP7-F/R and VP4-F/R primer pairs, adopted from the EuroRotaNet guidelines (www.eurorota.net) (Iturriza-Gomara et al., 2011). Amplification was carried out using the Superscript II One-step RT-PCR system (Life Technologies, Invitrogen Division). The RT and PCR conditions were set up according to the manufacturer's instructions, except for the annealing temperatures, which were 52 °C for VP7 and 50 °C for VP8*, as proposed by the EuroRotaNet guidelines. For genotyping, multiplex PCR was used separately for genotypes G (for the VP7 segment) and genotypes P (for the VP8* segment). For VP7, G1–G4, G8–G10, G12 and for VP4, P[4], P[6], P[8], P[9], P[10], P[11], type specific primers were included in a multiplex reaction (Iturriza-Gomara et al., 2011), using the *Tfi* polymerase enzyme system (Life Technologies, Invitrogen Division). Genotypes were confirmed based on the PCR amplified fragments, separated by 1.5% agarose gel electrophoresis.

2.3. Sequencing and NA analysis

The period of our study included rotavirus consecutive seasons 2005/06–2012/13. In each season, up to 20 rotavirus strains were selected with a representative number of genotypes: G1P[8], G2P[4], G4P[8] and G9P[8]. However, there were not enough strains in each season to reach this target for some genotypes. In total, 113 strains were analyzed: 31 G1P[8] (2 in 2005, 4 in each of 2006, 2007 and 2009–2012 and 5 in 2008); 21 G2P[4] (3 in 2007, 6 in each of 2008 and 2011, 1 in 2009, 2 in 2010, 3 in 2012); 33 G4P[8] (5 in each of 2005 and 2009, 4 in each of 2006, 2008 and 2010–2012, 3 in 2007), 28 G9P[8] (6 in 2005, 5 in 2012, 4 in 2006, 3 in each of 2007, 2009 and 2010, 2 in each of 2008 and 201).

For each rotavirus strain, VP7 and VP8* amplified fragments were purified using the Exo I/FastAP™ enzyme system (Thermo Fischer Scientific, Waltham, MA) (Werle et al., 1994). All genome fragments were directly sequenced with the initial PCR primers used in the first round RT-PCR of the VP7 and VP8* amplification reaction. An ABI PRISM BigDyeTM 3.1 terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) was used for the sequencing reaction in a 20 µl reaction and purified with a BigDye® Xterminator™ purification kit (Applied Biosystems). Sequence data were generated in an ABI 3500 Genetic Analyzer (Applied Biosystems).

Nucleotide sequences were aligned and assembled in CLC Main Workbench 6.9 software (CLC Bio, Aarhus, Denmark). Alignment and the construction of a Neighbor-Joining phylogenetic tree was performed in the MEGA 5.2 software package (Tamura et al., 2011).

Nucleotide and amino acid sequences obtained in this study were deposited in GenBank under accession numbers KJ432637–KJ432862.

2.4. Rotavirus disease burden and vaccination coverage in Slovenia

Epidemiological data of rotavirus infections were collected for children in the age group of 0–6 years for each of the analyzed season to estimate the eventual reduction of rotavirus disease. The overall rotavirus incidence was calculated based on the total reported cases of rotavirus infections (hospitalized and outpatients) and the hospitalization incidence was calculated according to the reported hospitalized cases of rotavirus diarrhea. The incidences were calculated for the total population of children in the age group 0–6 years.