

INTERNATIONAL MICROBIOLOGY (2016) 19:27-32
doi:10.2436/20.1501.01.260 ISSN (print): 1139-6709. e-ISSN: 1618-1095
www.im.microbios.org

Genetic and serologic surveillance of rotavirus with P[8] and P[4] genotypes in feces from children in the city of Chihuahua, northern Mexico

Juan F. Contreras-Cordero,^{1*} César I. Romo-Sáenz,¹ Griselda E. Menchaca-Rodríguez,¹ Rocío Infante-Ramírez,³ Licet Villarreal-Treviño,¹ Carlos E. Hernández-Luna,² Cristina Rodríguez-Padilla,¹ Reyes S. Tamez-Guerra¹

¹Department of Microbiology and Immunology, Faculty of Biological Sciences, Autonomous University of Nuevo Leon, San Nicolas de los Garza, Nuevo Leon, Mexico. ²Department of Chemistry, Faculty of Biological Sciences, Autonomous University of Nuevo Leon, San Nicolas de los Garza, Nuevo Leon, Mexico. ³Faculty of Chemical Sciences, Autonomous University of Chihuahua, Chihuahua, Mexico

Received 7 February 2016 · Accepted 7 March 2016

Summary. Rotavirus vaccine was developed using the most prominent G and P genotypes circulating in children population. Therefore, severe gastroenteritis has been reduced around the world. This study investigated the G and P rotavirus genotypes circulating in children from two hospitals in the city of Chihuahua, Mexico. Additionally, polyclonal antibodies against Rotavirus Wa strain were used to determine their homotypic and heterotypic reactivity to both P[8] and P[4] genotypes. G1, G2, and G3 VP7 genotypes and P[8] and P[4] VP4 genotypes were detected in common and uncommon combinations as well as mixed infectious. The predominant combination was G1P[8]. Phylogenetic analysis of VP4 gene revealed the presence of P[8]-1 and P[8]-3 lineages of P[8] genotype and P[4]-5 lineage of P[4] genotype. All but five G1P[8] rotavirus were detected by polyclonal anti-Rotavirus Wa strain. Mutation analysis revealed differences in three of the four neutralizing epitopes previously reported to VP8* subunit of VP4 protein. Results of this study offer insights over genetic variants of field rotavirus that could be detected in a homotypic and heterotypic way by antibodies elicited to rotavirus with P[8] genotype. [Int Microbiol 2016; 19(1):27-32]

Keywords: rotavirus · viral genotypes · lineages of virus · epitopes · Chihuahua, Mexico

Introduction

Rotavirus is the most important cause of viral gastroenteritis in humans. Annually, rotavirus is responsible for 197,000-233,000 deaths in children less than five years of age mainly

in developing countries [25]. Rotavirus (family *Reoviridae*, subfamily *Sedoreovirinae*, genus *Rotavirus*, species *Rotavirus A*) is a nonenveloped virion composed by six structural proteins that form three concentric layers enclosing a genome of 11 segments of double-stranded RNA. The outer layer is composed by VP7 and VP4 proteins [9]. Based on these proteins, rotavirus type A has been classified in a binary system. So far 27 G (VP7) and 37 P (VP4) genotypes have been identified [19,27].

Surveillance studies around the world, before and after the introduction of rotavirus vaccines have shown that P[8] is the main genotype circulating in human populations. To date,

*Corresponding author: Juan F. Contreras-Cordero
Department of Microbiology and Immunology
Faculty of Biological Sciences
Autonomous University of Nuevo Leon
San Nicolas de los Garza, Nuevo Leon, Mexico
Tel. +52-8183294115
E-mail: juan.contrerascr@uanl.edu.mx; contrerasjfc@gmail.com

P[8] genotype can be detected in levels of 70% of cases of infantile diarrhea, followed by P[4] that account nearly 15% [22]. Frequently, P[8] genotype is detected in combination with G1, G3, G4, G8 or G9 genotypes, whereas P[4] mainly is detected in combination with G2 genotype. In less extension, these P[8] and P[4] genotypes can also be detected in combination with other G genotypes [3,13,17].

P[8] and P[4] genotypes have been extensively studied in nucleotide and amino acid sequence. These studies have showed that genetic diversity is frequent among rotavirus circulating around the world. As a consequence, four lineages of P[8] (P[8]-1 to P[8]-4) and five lineages of P[4] (P[4]-1 to P[4]-5) have been detected with considerable intralinear and interlinear diversity [5,8,29]. Although P[8]-3 and P[4]-5 lineages are prevalent around the world, new lineages could emerge through different mechanisms including mutations, reassortment or recombination [1,20,23]. Nine antigenic regions have been established on the VP4 outer capsid protein that are responsible to elicit neutralizing antibodies. Four of these regions have been mapped on VP8* subunit (8–1 to 8–4) and five on VP5* subunit (5–1 to 5–5) [6,7]. Genetic studies have revealed changes in amino acid sequence on these epitopes among rotavirus strains with the same genotype and few studies focused on the combined effect of these changes on the detection of rotavirus by antibodies [1,15,29]. In this study, G and P genotypes of rotavirus circulating in the city of Chihuahua, Mexico, were detected. In addition, polyclonal antibodies elicited against Rotavirus Wa strain were used to test the reactivity of the lineages detected and to determine whether changes in the amino acid sequence of P[4] and P[8] genotypes could influence the antigen-antibody reaction.

Materials and methods

Stool specimens. A total of 140 positive samples for rotavirus detected by polyacrylamide gel electrophoresis (PAGE) collected from 2004 to 2011 from children younger than five years of age were used in this study. All samples were collected from Chihuahua General Hospital Dr. Salvador Zubiran and the Children's Hospital from the city of Chihuahua, State of Chihuahua, northern Mexico. The fecal specimens were stored at -20°C .

Reverse transcription polymerase chain reaction (RT-PCR). Viral RNA was purified from 20% stools suspension in phosphate-buffered saline (PBS) by using TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA) following the manufacturer's protocol. G and P genotypes were detected using the set of specific primers for G1-G4, P[8] and P[4] genotypes previously reported [10,11].

Sequencing and phylogenetic analysis of VP8* subunit. A PCR product of 762 bp of gene 4 using PA1 and PC2 primers was cloned and sequenced in the plasmid pGEM-T using the pGEM®-T Vector System I

(Promega, Madison, WI, USA) [4]. The nucleotide sequence was determined using an automated system (Perkin Elmer/Applied Biosystem, CA, USA) at the Institute of Biotechnology of the National Autonomous University of Mexico (IBT-UNAM). The sequence analysis was done using CLUSTALW/BioEdit sequence alignment Version 7.0 [12] and MEGA Version 6.0 software [24]. The VP4 nucleotide and amino acid sequences included in this study were submitted to the GenBank database under accession numbers JX012330 to JX012347. The GenBank accession numbers of VP4 gene used in sequence analysis were HQ585864 to HQ585866, FJ665380 to FJ6653091, M96825, JN849113, EU045252, and DQ492672.

Polyclonal antibodies. The whole Rotavirus Wa strain were used to produce polyclonal antibodies. Polyclonal antibodies against a mixture of whole Rotavirus strains Wa, DS-1, ST3, SA11 and YM, here denominated anti-RVs (kindly provided by Carlos F. Arias, IBT-UNAM, Mexico) were used to detect rotavirus in specimen samples.

Enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Costar, NY, USA) were coated with goat polyclonal anti-RVs diluted in carbonate-bicarbonate buffer pH 9.6 and incubated overnight at 4°C . After two washing with PBS, the plates were blocked with 5% non-fat dry milk in PBS at 37°C for 1.5 h. After four washes with PBS-Tween 0.1% (washing buffer), 20% fecal suspensions from children with gastroenteritis containing P[8] or P[4] rotavirus were added in duplicate. Fecal samples without rotavirus were used as negative controls. The plates were then incubated at 37°C for 2 h, and after washing four times, rabbit polyclonal anti-Rotavirus Wa strain was added and incubated at 37°C for 1 h. After four washes, peroxidase-conjugated protein A (Amersham, Buckinghamshire, UK) was added and incubated at 37°C for 1 h. The plates were then washed four times and the ABTS (2,2'-azino-bis[3-ethylbenzothiazoline-6-sulphonic acid]) peroxidase substrate (KPL, Gaithersburg, MD, USA) was added and incubated at 37°C for 30 min. The absorbance at 405 nm was measured using an automatic microplate reader (Digital and Analog Systems, Roma, Italy). The cutoff value was defined as the mean of the control negative OD 405 nm values plus three standard deviations.

Results

G and P genotyping. Of the total samples, two specimens positive by electrophoresis were typed as rotavirus group C, and 138 (98.5%) were assigned as G and P genotypes, respectively. Of the latter, both genotypes were detected in 128 (92.7%). The most prevalent combination was G1P[8] with 57.85%, and G2P[4] genotype was detected in a small proportion (2.86%) of the specimens tested. However, either P[8] or P[4] were found in 133 specimens including samples with mixed infections of both genotypes (Table 1).

VP4 lineages. To determine the genetic diversity of rotavirus belonging to the VP4 genotypes detected, 33 VP4 genes of the VP8* subunit were analyzed. Of these, 25 samples of P[8] genotype grouped into two lineages; 5 of them had 97.2–98% identity with the Wa strain of P[8]-1 lineage, and 20 had 98–99.6% identity with the Dhaka 16-03 Bangladesh strain of P[8]-3 lineage. Identity of P[8]-1 and P[8]-3 lineages used in

Table 1. Prevalence of VP7 and VP4 rotavirus genotypes in feces from 140 positive samples from children under five from two hospitals in the city of Chihuahua, Mexico

| G and P-type | Strain | N | % |
|--------------------|--------------|-----|-------|
| Common types | | | |
| | G1P[8] | 81 | 57.85 |
| | G3P[8] | 5 | 3.57 |
| | G2P[4] | 4 | 2.86 |
| Uncommon types | | | |
| | G1P[4] | 12 | 8.57 |
| | G2P[8] | 1 | 0.71 |
| | G3P[4] | 1 | 0.71 |
| Mixed infections | | | |
| | G1P[8]P[4] | 17 | 12.14 |
| | G2P[8]P[4] | 2 | 1.43 |
| | G3P[8]P[4] | 4 | 2.86 |
| | G1G3P[8]P[4] | 1 | 0.71 |
| G or P non typable | | | |
| | NEGP[8]P[4] | 1 | 0.71 |
| | NEG P[8] | 4 | 2.86 |
| | G1 NEG | 5 | 3.57 |
| Group C | | | |
| | NEG NEG | 2 | 1.43 |
| Total | | 140 | 100 |

this study ranged from 93.6 to 91.6% (Fig. 1). In turn, eight P[4] rotavirus grouped into the P[4]-5 lineage with high identity to each other, ranging from 98.8 to 100% identity with the PY05SR1297 Paraguay strain of P[4]-5 lineage (Fig. 1). Identity of amino acids between P[8] and P[4] lineages was in order of 85.6 to 90%.

ELISA of field rotavirus. To evaluate the antigenicity of genotypes detected, 94 fecal samples positive to Rotavirus type A with sufficient material were further subjected to ELISA. Both P[4] and P[8] genotypes were present in the fecal specimens. Of the 94 fecal specimens, P[8] was present in 63 (67%), P[4] in 18 (19.1%), and mixed P[4] and P[8] in 13 (13.9%). All specimens carried P[4] were detected, however, all but 5 (5.3%) specimens carried G1P[8] genotype were detected by these antibodies (Table 2).

Comparison of P[8] and P[4] antigenic regions of VP8* subunit. The genetic variation into rotavirus was made using 33 samples randomly selected. This comparison

was made with the prototype Rotavirus Wa strain and the Rotarix vaccine. The study of the 33 sequences analyzed revealed 58 variations in the VP8* subunit of VP4 when they were compared with VP8* sequence of Rotavirus Wa strain. The analysis of all changes showed that 37 amino acid substitutions were between residues with similar properties. In addition, 15 charge amino acid changes (Y19H, E28K, T75K, N89D, N113T/D/S, D116N, S131R/E, D133E/S, D135N, N160D, N193D, C215R, K245R/T/N, N250K and E251K) were identified as well as 6 changes of residues with possible structural implications (conformational flexibility or constrain) on the proteins (G38S, P71S, P114Q, G145S, G195N and P236S). Specific comparison of the amino acid residues in antigenic epitopes of VP8* subunit between Rotavirus Wa and P[8] and P[4] genotypes of field rotavirus showed differences on 3 of the 4 epitopes. In addition, the analyses on the epitope 8-1 and 8-3 identified 5 and 8 changes respectively, while rotavirus with P[4] genotype had differences on the epitope 8-4. The alignment between Rotavirus Wa and Rotarix vaccine revealed consensus in the antigenic domains of the VP8* subunit.

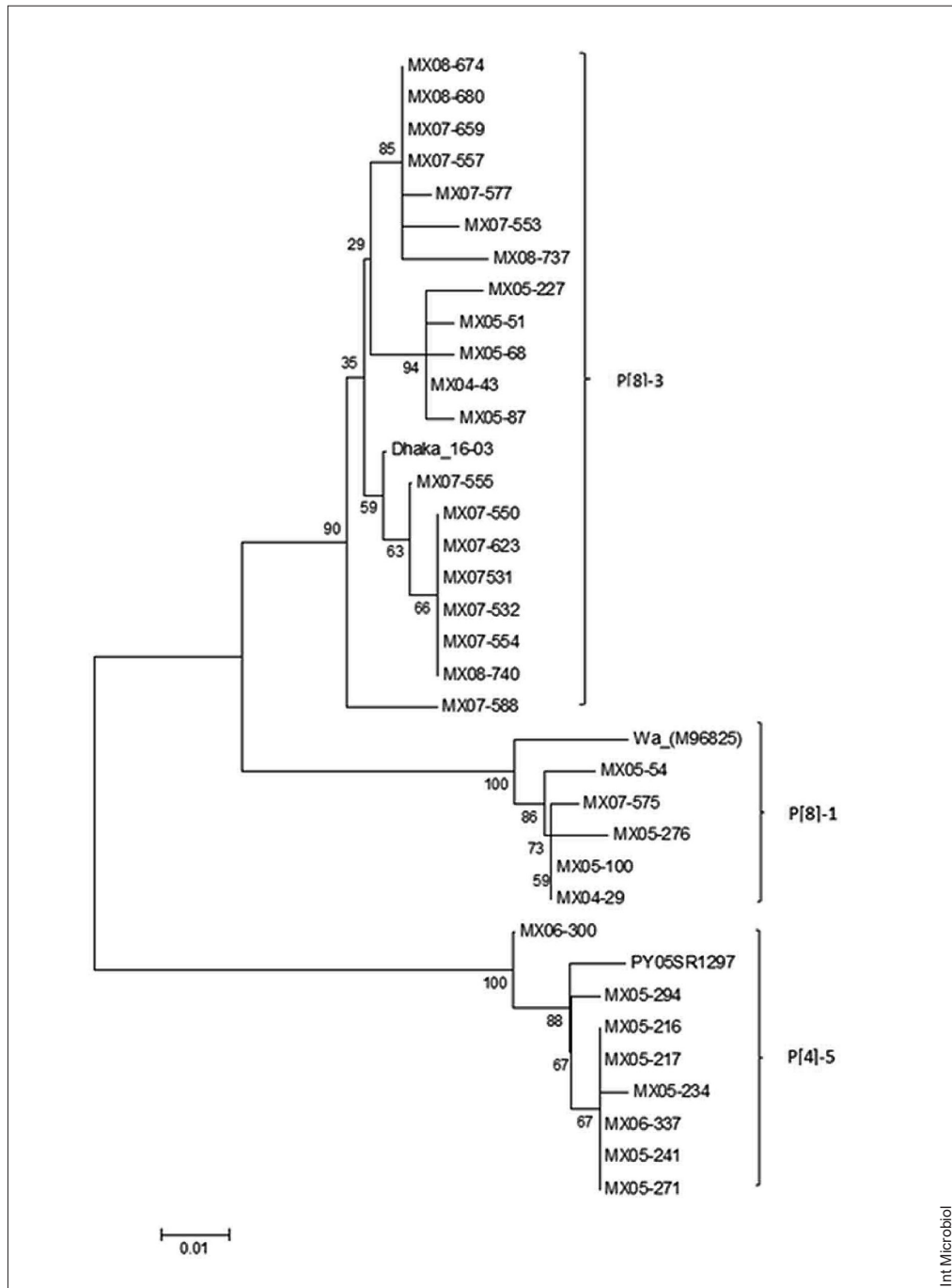


Fig. 1. Phylogenetic tree constructed from VP8* subunit amino acid sequences of the P[8] and P[4] rotavirus genotypes using neighbor-joining method (MEGA v6.0) with bootstrap analysis of 1000 replicates. Stool specimens selected from 140 positive samples for rotavirus from children under five from two hospitals in the city of Chihuahua, Mexico. Bar indicates 1% amino acid difference.

Discussion

Traditionally, epidemiological studies based on surface proteins VP7 and VP4 have classified rotavirus in a binary sys-

tem, identifying multiple genotypes combinations G/P [19]. Despite the great variability of this pathogen, G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] genotypes have been frequently detected around the world and G12P[8] genotype in a lesser extension [13,17,21]. In this study, P[8] and P[4] genotypes


Table 2. Rotavirus genotyped detected by polyclonal antibodies anti-Rotavirus Wa strain and enzyme-linked immunosorbent assay (ELISA)

| Genotype | Anti-Rotavirus Wa strain | |
|------------|--------------------------|------------|
| | Positive | Negative |
| G1P[8] | 56 | 5 |
| G1P[4] | 11 | 0 |
| G2P[4] | 5 | 0 |
| G3P[8] | 2 | 0 |
| G3P[4] | 1 | 0 |
| G1P[8]P[4] | 12 | 0 |
| G2P[8]P[4] | 1 | 0 |
| NEGP[4] | 1 | 0 |
| Total | 89 (94.68 %) | 5 (5.32 %) |

were detected in more than 95% of the rotavirus analyzed, indicating the same prevalence of these genotypes worldwide [1,13,23]. In addition, phylogenetic analysis of P[8] and P[4] genotypes have shown four and five lineages respectively. Of them, P[8]-3 and P[4]-5 lineages are prominent around the world [5,8,15,23]. In this study lineage P[8]-3 was detected in 80% of rotavirus with P[8] genotype and, to a lesser extent, in the lineage P[8]-1, which corresponds to the lineage present in the Rotarix vaccine. Moreover, lineage P[4]-5 was identified in all samples with P[4] genotype, which corresponds to the lineage of this genotype with more incidence worldwide [8,26]. After the introduction of the vaccine, several reports have revealed the need of continued epidemiological surveillance of rotavirus to understand the impact of genetic variation in the efficiency of the vaccine [2,16,18,23,29].

In this study, 94.7% of the samples analyzed were recognized by antibodies against Rotavirus Wa strain. These results are in accordance with previous reports that showed homotypic and heterotypic antibody reactivity between strains with different genotype [28]. Note that all rotavirus with P[4] genotype were recognized by these antibodies. However, five with P[8] genotype did not show reactivity, which suggests that amino acid changes in regions of VP8* subunit could interfere with antibody recognition and consequently could generate strains of rotavirus that elude the immunity generated by vaccination. The sequence analysis conducted in this study revealed both amino acid changes with similar properties and amino acid changes that could be related to physicochemical and structural alterations in the VP8* subunit.

Likewise, the analysis of variations between the sequenc-

es analyzed revealed that residues from 106 to 199 had the majority of amino acid changes of VP8*. Note that, in this region, there are epitopes responsible for neutralizing antibodies previously reported [6,7]. However, despite amino acid changes present in the VP8* subunit, these antibodies maintained the reactivity in the majority of the specimens. Sequence analysis of two negative samples by ELISA showed one specific amino acid substitution (N113T), when they were compared with vaccine strain or genetic variants (N113D/S) of positive samples, specifically into the epitope 8-3 on the VP8* subunit. This result is in contrast with previous reports that have revealed that single amino acid substitutions in these regions can alter the antigenic characteristics of VP4 [14]. Therefore, it is necessary to extend the studies of antigenic variability in field rotavirus to establish the possible role of putative surface residues as N/D/S at 113 position as well as genetic variants as N113T or other residues with conformational implications to elude antibody recognition. 

Acknowledgements. This work was supported in part by grant CN1074-11 PAICYT from the Autonomous University of Nuevo Leon, Mexico. César I. Romo-Sáenz is recipient of a fellowship from the CONACYT, Mexico.

Competing interests. None declared.

References

1. Afrad MH, Hassan Z, Farjana S, Moni S, Barua S, Das SK, Faruque AS, Azim T, Raham M (2013) Changing profile of rotavirus genotypes in Bangladesh, 2006-2012. *BMC Infect Dis* 13:320-326
2. Alam MM, Khurshid A, Shaikat S, Suleman RM, Sharif S, Angez M, Malik SA, Ahmed TM, Aamir UB, Naeem M, Zaidi SS (2013) Epidemiology and genetic diversity of rotavirus strains in children with acute gastroenteritis in Lahore, Pakistan. *PLoS One* 8:e67998
3. Chieochansin T, Vutithanachot V, Phumpholsup T, Posuwan N, Theamboonlers A, Poovorawan Y (2016) The prevalence and genotype diversity of Human Rotavirus A circulating in Thailand, 2011-2014. *Infect Genet Evol* 37:129-136
4. Contreras JF, Menchaca GE, Infante R, Hernández CE, Rodríguez C, Tamez RS (2011) Evidence of conserved epitopes in variable region of VP8* subunit of VP4 protein of rotaviruses of P[8]-1 and P[8]-3 lineages. *Acta Virol* 55:273-278
5. da Silva FM, Gómez MM, Rose TL, Volotão EdeM, Carvalho-Costa FA, Bello G, Leite JP (2013) VP8* P[8] lineages of group A rotaviruses circulating over 20 years in Brazil: Proposal of six different sub-lineages for P[8]-3 clade. *Infect Genet Evol* 16:200-205
6. Dormitzer PR, Nason EB, Prasad BV, Harrison SC (2004) Structural rearrangements in the membrane penetration protein of a non-enveloped virus. *Nature* 430:1053-1058
7. Dormitzer PR, Sun ZY, Wagner G, Harrison SC (2002) The rhesus rotavirus VP4 sialic acid binding domain has a galectin fold with a novel carbohydrate binding site. *EMBO J* 21:885-897

8. Espínola EE, Amarilla A, Arbiza J, Parra GI (2008) Sequence and phylogenetic analysis of the VP4 gene of human rotaviruses isolated in Paraguay. *Arch Virol* 153:1067-1073
9. Estes MK, Greenberg HB (2013) Rotaviruses. In: Knipe DM, Howley PM (eds) *Fields Virology*, 6th ed. Williams & Wilkins, Philadelphia, PA, USA, pp 1347-1401
10. Gentsch JR, Glass RI, Woods P, Gouvea V, Gorziglia M, Flores J, Das BK, Bhan MK (1992) Identification of group A Rotavirus gene 4 types by polymerase chain reaction. *J Clin Microbiol* 30:1365-1373
11. Gouvea V, Glass RI, Woods P, Taniguchi K, Clark HF, Forrester B, Fang ZY (1990) Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. *J Clin Microbiol* 28:276-282
12. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/ NT. *Nucleic Acids Symp* 41:95-98
13. Hungerford D, Vivancos R, EuroRotaNet network members, Read JM, Pitzer VE, Cunliffe N, French N, Iturriza-Gómara M (2016) In-season and out-of-season variation of rotavirus genotype distribution and age of infection across 12 European countries before the introduction of routine vaccination, 2007/08 to 2012/13. *Euro Surveill* 21:30106
14. Kirkwood CD, Bishop RF, Coulson BS (1996) Human rotavirus VP4 contains strain-specific, serotype-specific and cross-reactive neutralization sites. *Arch Virol* 141:587-600
15. Kulkarni R, Arora R, Arora R, Chitambar SD (2014) Sequence analysis of VP7 and VP4 genes of G1P[8] rotaviruses circulating among diarrhoeic children in Pune, India: A comparison with Rotarix and RotaTeq vaccine strains. *Vaccine* 32:A75-A83
16. László B, Kónya J, Dandár E, Deák J, Farkas Á, Gray J, Grósz G, Iturriza-Gómara M, Jakab F, Juhász A, Kisfali P, Kovács J, Lengyel G, Mattella V, Melegh B, Mészáros J, Molnár P, Nyúl Z, Papp H, Pátri L, Puskás E, Sántha I, Schneider F, Szomor K, Tóth A, Tóth E, Szucs G, Bányai K (2012) Surveillance of human rotaviruses in 2007–2011, Hungary: Exploring the genetic relatedness between vaccine and field strains. *J Clin Virol* 55:140-146
17. Mandile MG, Esteban LE, Argüelles MH, Mistchenko A, Glikmann G, Castello AA (2014) Surveillance of group A Rotavirus in Buenos Aires 2008-2011, long lasting circulation of G2P[4] strains possibly linked to massive monovalent vaccination in the region. *J Clin Virol* 60:282-289
18. Gómez MM, Carvalho-Costa FA, Volotão EdeM, Rose TL, da Silva MF, Fialho AM, de Assis RM, Matthijnssens J, Leite JP (2014) A decade of G3P[8] and G9P[8] rotaviruses in Brazil: Epidemiology and evolutionary analyses. *Infect Genet Evol* 28:389-397
19. Matthijnssens J, Ciarlet M, McDonald SM, Attoui H, Bányai K, Brister JR, Buesa J, Esona MD, Estes MK, et al (2011) Uniformity of Rotavirus Strain Nomenclature Proposed by the Rotavirus Classification Working Group (RCWG). *Arch Virol* 156:1397-1413
20. Matthijnssens J, Van Ranst M (2012) Genotype constellation and evolution of group A rotaviruses infecting humans. *Curr Opin Virol* 2:426-433
21. Mijatovic-Rustempasic S, Teel EN, Kerin TK, Hull JJ, Roy S, Weinberg GA, Payne DC, Parashar UD, Gentsch JR, Bowen MD (2014) Genetic analysis of G12P[8] rotaviruses detected in the largest U.S. G12 genotype outbreak on record. *Infect Genet Evol* 21:214-219
22. Santos N, Hoshino Y (2005) Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine. *Rev Med Virol* 15:29-56
23. Steyer A, Sagadin M, Kolenc M, Poljšak-Prijatelj M (2014) Molecular characterization of rotavirus strains from pre- and post-vaccination periods in a country with low vaccination coverage: The case of Slovenia. *Infect Genet Evol* 28:413-425
24. Tamura K, Stecher G, Peterson D, Filipiński A, Kumar S (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 30:2725-2729
25. Tate JE, Burton AH, Boschi-Pinto C, Parashar UD, World Health Organization-Coordinated Global Rotavirus Surveillance Network (2016) Global regional, and national estimates of rotavirus mortality in children >5 years of age, 2000–2013. *Clin Infect Dis* 62 Suppl 2:S96-S105
26. Tatte V, Chitambar SD (2011) Intragenotypic diversity in the VP4 encoding genes of rotavirus strains circulating in adolescent and adult cases of acute gastroenteritis in Pune, Western India: 1993 to 1996 and 2004 to 2007. *J Gen Mol Virol* 3:53-62
27. Trojnar E, Sachsenröder J, Twardziok S, Reetz J, Otto PH, Johne R (2013) Identification of an avian group A rotavirus containing a novel VP4 gene with a close relationship to those of mammalian rotaviruses. *J Gen Virol* 94:136-142
28. Wen X, Cao D, Jones RW, Hoshino Y, Yuan L (2015) Tandem truncated rotavirus VP8* subunit protein with T cell epitope as non-replicating parenteral vaccine is highly immunogenic. *Hum Vaccin Immunother* 11:2483-2489
29. Zeller M, Patton JT, Heylen E, De Coster S, Ciarlet M, Van Ranst M, Matthijnssens J (2011) Genetic analyses reveal differences in the VP7 and VP4 antigenic epitopes between human rotaviruses circulating in Belgium and rotaviruses in Rotarix and RotaTeq. *J Clin Microbiol* 50:966-976