UNIVERSIDAD SAN FRANCISCO DE QUITO

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Rápido reemplazo de genotipo rotaviral en Ecuador

Rapid succession of rotaviral genotypes in Ecuador

(*el idioma de esta tesis es inglés*)

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Resumen

Un estudio previo indicó que G9P[8] fue el genotipo de rotavirus más prevalente en 22 comunidades rurales de Esmeraldas y un hospital urbano de Quito, Ecuador durante febrero 2005- febrero 2006. El objetivo del presente estudio fue caracterizar los genotipos rotavirales que circularon las mismas áreas rurales durante los años 2006-2007, y el mismo hospital urbano durante el año 2007. En total 990 muestras de heces fueron recolectadas en las áreas rurales, de las cuales 84 (74 casos y 14 controles) fueron rotavirus positivas a un test inmunocromatográfico. La genotipificación mediante RT-PCR de todas las muestras positivas demostró que durante 2006 el genotipo más común fue G9 (22%); mientras que en 2007 fue G2 (34%). Las muestras del hospital urbano también demostraron un predominio de G2 (53%) vs. G9 (27%). Tanto en las muestras urbanas como rurales G2 se encontró asociado principalmente con P[4]. Una proporción considerable de muestras rurales (58%) no pudieron ser genotipificadas, posiblemente como consecuencia de una gran variabilidad genética del virus en esta zona. El rápido reemplazo de G9 por G2 y la gran cantidad de aislados rurales no genotipificados refuerza la necesidad de establecer sistemas de vigilancia que provean información precisa a programas de vacunación contra rotavirus.

Abstract

A previous study indicated that G9P[8] was the most prevalent rotavirus genotype in 22 rural communities of Esmeraldas and an urban hospital in Quito, Ecuador during February 2005- February 2006. The objective of the present study was to characterize the rotavirus genotypes that circulated the same rural areas during the years 2006 - 2007 and the same urban hospital during the year 2007. A total of 990 stool samples were collected in the rural areas, of which 84 (70 from cases and 14 from controls) were rotavirus positive by an immunochromatographic test. Genotyping of all rotavirus positive samples by RT-PCR showed that the most common typeable genotype was G9 (22%) in 2006, but G2 (34%) in the following year. The samples from the urban hospital also showed a predominance of G2 (53%) vs. G9 (27%). In the urban as well as the rural samples, G2 was mainly associated to P[4]. A large number of rural samples (58%) could not be genotyped, possibly as consequence of a high genetic variability of the virus in that area. The sudden replacement of G9 by G2 and the high number of rural untypeable isolates reinforces the necessity of establishing surveillance programs to supply accurate information to vaccination programs against rotavirus.

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1. Introduction

Rotavirus is the main diarrhea causing agent responsible for 454,000-705,000 children deaths each year worldwide (1). The virus belongs to the *Reoviridae* family; it has a three layered capsid that surrounds a genome made of 11 segments of double stranded RNA. The middle capsid layer is composed of the VP6 protein which is the most abundant protein of the virus and permits classification into groups A to E based on the presence of specific epitopes. Most human infecting rotaviruses belong to group A (2).

Proteins VP7 and VP4, also named G and P respectively, conform the outermost layer of the capsid. Both proteins are neutralizing immunogens, and the nucleotide sequences of the corresponding genes are utilized for typing purposes The Rotavirus Classification Working Group has catalogued 19 VP7 genotypes and 27 VP4 genotypes (3), and a novel genotype (G20P[28]) was recently described in Ecuador (4). Globally, G1, G2, G3, G4, and G9 are the most predominant genotypes of VP7, whereas P[8], P[4], P[6]and P[9] are the most prevalent of VP4 (5, 6). There are uncommon genotypes like G5, G6, G8, and G10 that are endemic in certain communities in Brazil, Australia, Malawi, and India respectively (5, 6).

Rotavirus has a large intra and inter genotype diversity because of genetic reassortments, point mutations, genomic rearrangement, and genetic recombination (5, 7). This diversity constitutes an important problem for the design of effective vaccines to decrease rotavirus associated mortality and morbidity. Although available rotaviral vaccines have shown to protect against severe gastroenteritis caused by genotypes G1, G2, G3, G4 and G9 (8, 9), it is still uncertain the degree of cross protection against genotypes not present in vaccines (9, 10, 11).

A previous study on rotavirus genotype prevalence conducted recently in Ecuador showed that G9 was the most prevalent rotavirus genotype circulating in 22 rural communities of Esmeraldas and a children's hospital in Quito during February 2005-February 2006 (12). The present study constitutes a follow up study of the previous one and therefore covered the same areas during the next two subsequent years (2006 and 2007). Differently from most studies of rotavirus genotype prevalence, which are based on hospital samples, the results from the rural settings obtained in this study are community based and included both asymptomatic and symptomatic patients. The hospital samples from a distant urban location, Quito, were used as an indicator of the representativity of the results obtained in rural areas.

2. Materials and Methods

2.1. Study Population and Design

All protocols have been described elsewhere (12). Briefly, twenty-two rural communities located in the northern coast of Ecuador, were visited two times for 15 days, first between May and December, 2006, and the second time between January and July, 2007. Each household was visited in order to capture all cases of diarrhea. For each case of diarrhea, three additional stool specimens were randomly collected from controls, one from the patient household and two randomly selected from the same community. Cases were defined as individuals with three or more loose stools in a 24h period and controls as individuals with no diarrhea within the past 6 days. Rotavirus positive fecal samples from the Hospital de Niños Baca Ortiz in Quito were also collected between January 2007 and May 2007 for PCR genotyping. All protocols were approved by the IRB committees of the University of Michigan and Universidad San Francisco de Quito.

2.2. Detection of Rotavirus and RNA extraction

All fecal samples were tested for rotavirus with the RIDA Quick Rotavirus immunochromatographic test (R-Biopharm AG, Darmstadt, Germany), stored in liquid nitrogen, and sent to Quito for PCR-genotyping. RNA from all rotavirus positive samples was extracted with the kit QIAamp Viral RNA Mini kit (Qiagen, Germany) and RNA was stored at -80°C for further analysis (12).

2.3. RT-PCR and Multiplex-PCR for rotavirus genotyping

Rotavirus genotypes were determined with a two step seminested multiplex reverse transcription PCR as described elsewhere (12). After viral RNA was denatured at 97°C for

5 min., retro-transcription and first amplification were carried out using SuperScript III RT/Platinum Taq polymerase kit (Invitrogen Corp.). Primers were added to a final concentration of 267nM each, 9Con1 (forward) and 9Con2 (reverse) for the VP7 amplification (13) or Con3 (forward) and Con2 (reverse) for the VP4 amplification (14). Retro-transcription was carried out at 42°C for 45 min and stopped at 96°C for 2 min. First amplification cycling parameters were 30 cycles at 94°C for 30s, 50°C for 30s, and 72°C for 60s.

Genotypes G and P were determined in a second amplification by using PuReTaq Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ, USA) and primers at a final concentration of 400nM. For G genotyping, primer 9Con1 and reverse primers 9T-1, 9T-2, 9T-3P, 9T-4 and 9T-9B (13) were used. Primer Con3 and reverse primers 1T-1, 2T1, 3T-1, 4T-1, 5T-1 y ND3 (14) were used for P genotyping. The cycling parameters were 30 cycles at 94°C for 30s, 42°C for 30s, and 72°C for 60s and final extension at 72°C for 1 min. The amplicons of the second amplification were run on a 2% agarose electrophoresis gel and bands were visualized by ethidium bromide staining.

2.4. Statistical analysis

Descriptive statistics were used to present all results. Means were calculated for continuos variables and percentages were calculated for categorical variables. In order to compare differences in rotavirus presence among cases and controls, the odds ratio was calculated along with its 95% confidence interval which was calculated through Fisher's chi square test. P values ≤ 0.05 were considered statistically significant.

3. Results

A total of 990 stool samples were collected in two visits to 22 rural communities of the northern coast of Ecuador, 281 (28.4%) of these samples were from patients with diarrhea and 709 (71.6%) from asymptomatic controls. The immunochromatographic test showed that 84 (8.5%) samples were rotavirus positive, of which 70 (7.1%) were from patients with diarrhea while the remaining 14 (1.4%) samples were from asymptomatic controls. The presence of diarrhea was significantly associated with rotavirus infection (odds ratio=16.5; 95% confidence interval 8.9 to 32.2) (Table 1). Rotavirus prevalence on a 15 day period had the highest value (9.8 %) for children under one year of age (Table 1).

Table 1. Number of rotavirus positive samples by age group

	Age group						
	Missing birthdates	**<1**	**1 - <5**	**5 - <20**	**20 - <40**	**>40**	**Total**
Cases (+/n)	10/32	19/43	23/128	10/43	2/12	6/23	70/281
Controls (+/n)	1/48	0/15	2/69	5/294	4/136	2/147	14/709
OR (95%CI)	---	∞	7,3 (1,7-65,8)	17,5 (5,0-68,3)	6,6* (0,5-51,8)	25,6 (4,0-268,0)	16,5 (8,9-32,2)
Prevalence (%)**	2,7	9,8	2,3	0,4	0,3	0,4	1,0

+ indicates number of rotavirus positive samples with the immunochromatographic test. OR, odds ratio; CI, confidence interval. The CI was calculated using the Fisher exact test.

*Not significative (P>0.05).

**Prevalence on 15 days, obtained from a mean of rotavirus cases per visit.

A large proportion (58%) of these samples was untypeable for VP4 and VP7 genes (Table 2). Another group of rotavirus positive samples could not be completely genotyped, 15 yielded results only for VP7 and one only for VP4. Among the typeable samples, G9 was the most prevalent genotype during 2006 (22%) followed by genotype G2 (7%). However

in samples from 2007, G2 was the most prevalent typeable genotype representing 34% of all rotavirus positive samples, followed by G9, which accounted for 7%. In all community samples there was no evidence of mixed genotype infection, but two hospital samples showed two different G or P types in the same sample. The most frequent G and P combinations detected in this study were, G2P[4] and G9P[8]. A separated analysis of control and case-patient samples shows that the most prevalente genotypes, that is G2, G9 and the combinations G2P[4] and G9P[8], were detected in controls as well as case-patient (Table 3).

Table 2. Rotavirus Genotype Combinations detected in stool samples from the rural coastal communities and the children hospital in Quito between 2006 and 2007

	Number of strains detected (%)		
	Coastal communities		Quito
Genotype	**2006, first visit**	**2007, second visit**	**2007**
G1 P_{NT}	3(5,6)	-	-
G2 P_{NT}	1(1,9)	1(3,4)	1(3,3)
G9 P_{NT}	10(18,5)	-	1(3,3)
G_{NT} P[4]	1(1,9)	-	-
G1 P[6]	1(1,9)	-	-
G3 P[6]	1(1,9)	-	-
G9 P[4]	1(1,9)	-	-
G9 P[8]	1(1,9)	2(6,9)	8(26,7)
G2 P[4]	3(5,6)	9(31,0)	16(53,3)
G4 P[6]	-	-	2(6,7)
G20 P[28]	1(1,9)	-	-
G9 P[8]/P[6]	-	-	1(3,3)
G4/G9 P[6]	-	-	1(3,3)
G_{NT} P_{NT}	31(57,4)	17(58,6)	-
TOTAL	54	29	30

NT = not typeable

A subset of 30 samples randomly selected form 102 rotaviral positive samples collected from children at the Hospital de Niños Baca Ortiz in 2007, was genotyped by RT-PCR. Most of the samples (28 out of 30) were successfully genotyped for both genes; two

samples could not be genotyped for the P segment. The predominant genotype was G2P[4] accounting for 53,3% of all samples while G9P[8] occurred in 26,7% of the samples. Two cases of mixed infections were found among the hospital samples (Table 2).

Table 3. Rotavirus Genotype Combinations detected in stool samples from the rural coastal communities between 2006 and 2007 on cases and controls

	Number of strains detected	
Genotype	**Cases (%)**	**Controls (%)**
G1 P_{NT}	3(4,3)	-
G2 P_{NT}	1(1,4)	1(7,1)
G9 P_{NT}	8(11,4)	2(14,3)
G_{NT} P[4]	1(1,4)	-
G1 P[6]	1(1,4)	-
G3 P[6]	1(1,4)	-
G9 P[4]	1(1,4)	-
G9 P[8]	2(2,9)	1(7,1)
G2 P[4]	11(15,7)	1(7,1)
G20 P[28]	1(1,4)	-
G_{NT} P_{NT}	40(57,1)	9(64,3)
TOTAL	70(100)	14(100)

4. Discussion

The present results show evidence of an abrupt replacement of genotype G9 with G2 in two different regions of Ecuador within a two year period. According to a previous study carried out in the same locations during 2005-2006 (12), the most prevalent genotype detected in the rural communities and Quito, was G9 with proportions of 72% and 90% respectively, while G2 was not found in the 22 rural communities and was a minor genotype in samples from the urban hospital. A more recent study carried out in Ecuador by Naranjo *et al*., reported that during 2006 G9 was the most prevalent genotype found in 10 different provinces of Ecuador (Azuay, Cotopaxi, Chimborazo, Imbabura, Pichincha, Esmeraldas, Guayas, Manabí, Napo and Pastaza) followed by G2 which was found in approximately 28% of the samples. (15). In the present study it was shown that G2 infection went from 7% in 2006 to 34% in 2007 within the rural settings, and it reached 57% in the urban hospital during 2007. Along with this rise on G2 prevalence, a decrease of G9 was observed also at both locations.

Other studies have also shown that the prevalent rotavirus genotypes circulating within a population change in short periods of time (16, 17). In Chiang Mai, Thailand, during the years 2000-2001, the most prevalent genotype was G9; by the year 2003, G2 emerged as the prevailing genotype but only to decline again in 2004, when G1 became the most common (16). In Bangladesh a change of the prevailing genotypes, similar to the change reported in this study, has also been observed: between years 2001 and 2005 the prevailing strains belonged to genotypes G1P[8] and G9P[8], however by the 2005-2006 season G2P[4] became predominant (17)

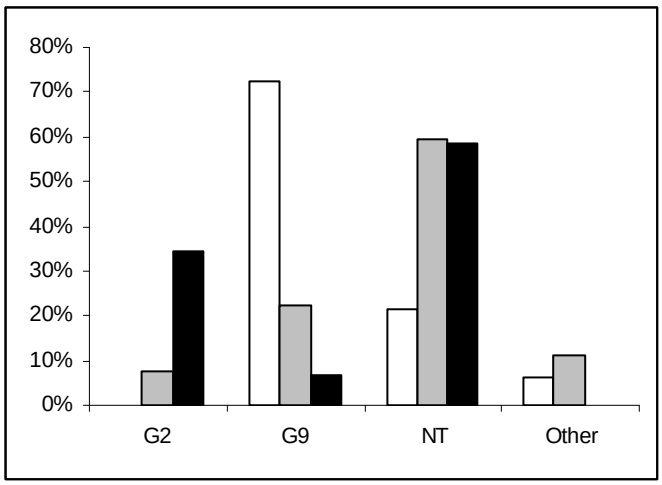
Changes in the pattern of prevailing viral genotypes within a population may be explained by the interplay between population's immunity and viral evolution. The presence of antibodies against a serotype of rotavirus prevent re-infections by the antigenically related viruses but may not against other distinct serotypes (2, 5, 10, 18). The population may build up immunity reducing the circulation of G9 and allowing antigenically distinct serotypes to infect. Additionally, viral variants possessing greater infective aptitudes may emerge only to disappear when populations reach a certain level of herd immunity (19).

The large proportion of samples from rural communities that could not be genotyped constitutes a limitation of this study. Many studies of rotavirus surveillance have reported different rates of genotyping failure e.g. 16% in Western Africa (20), ≤2% in Denmark and Malawi (21, 22), 0% in Bangladesh and Thailand (16, 17). In Ecuador, Naranjo *et al* and Endara *et al* report a similar rate of genotyping failure (18% and 17% respectively) (12, 15)

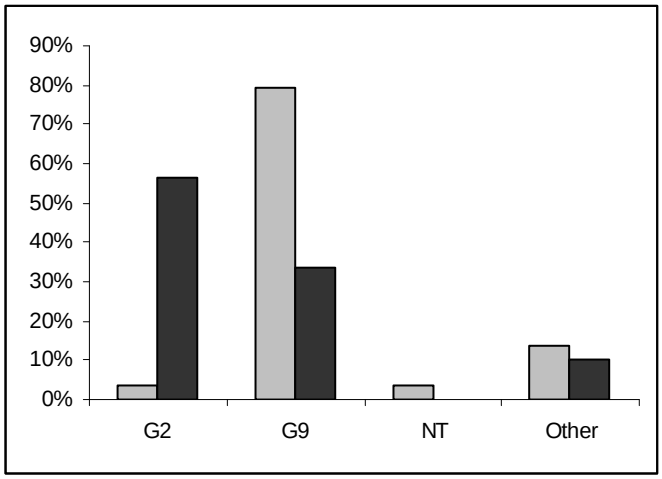
The increased rate of genotyping failure observed within rural communities could be due the presence of unusual strains, which often occur in developing countries and they are likely to infect humans due to contact with animals (5, 6). These zoonotic infections promote the emergence of new genotypes by viral reassortment. The presence of unusual genotypes may explain the greater abundance of what appeared to be untypeable virus in samples from rural communities when compared with samples from urban setting. An evidence that supports this theory is that one of the isolates found during this study was a rotavirus with novel G and P types (4). This would suggest that before 2007 the G9 genotype was able to temporarily supplant indigenous novel strains, explaining the higher proportion of isolates that could be typed during 2005 (Figure 1).

Figure 1. Comparation of percent isolates identified in the rural communities of Esmeraldas and Quito as genotype G2, G9 by year

A



B



White: 2005, grey: 2006, black: 2007. A) Esmeraldas; B) Quito. NT designates undetermined types. 'Other' includes genotypes G1, G3, and G4. The data from 2005 was taken from Endara *et al*, 2007 (12)

Other causes of genotyping failure may be sample degradation, the presence of false positive results in the immunochromatographic test, primer-template mismatch because of minor sequence variations (23; 24; 25) or primer competition during nested PCR (26).

However these explanations are undermined by two facts: first, stability tests conducted with fecal samples (4) and a previous study (27) suggesting that rotaviral particles are very stable and; second, genotyping failure was observed almost exclusively in the rural samples thus, if the problem was the procedure then a similar percentage of genotyping failure would have been found among urban samples as well.

The impact that vaccination programs may have on this study is none or not significant because a vaccine against rotavirus have been administrated freely in Ecuador only after July 2007 (28) and by this time all the samples analyzed in this study were already collected. Before July 2007 vaccines were offered in the country but only by pediatricians on private consultation and at a relatively expensive price (29).

To date, no rotaviral vaccine has proved to be adequately effective against G and P protein types not present their formulations. The sudden change of G9 by G2 found in this study, and the presence of a considerable high number of untypeable strains in rural areas reinforce the need to establish permanent rotaviral surveillance studies to give accurate information to vaccination programs.

5. References

1. Parashar UD, Gibson CJ, Bresee JS, Glass RI. Rotavirus and severe childhood diarrea. Emerg Infect Dis. 2006 Feb;12(2):304-6.

2. Estes MK, Kapikian AZ. Rotaviruses. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, editors. Fields Virology. Philadelphia: Lippincott Williams & Wilkins; 2007. p 1917-74.

3. Matthijnssens J, Ciarlet M, Rahman M, Attoui H, Banyai K, Estes MK, et al. Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments. Arch Virol. 2008 Aug;153(8):1621-9.

4. Solberg O, Hasing M, Trueba G, Eisenberg J. Characterization of novel VP4, VP6, and VP7 genotypes of a previously untypeable group A rotavirus. Virol J. In Press 2008.

5. Gentsch JR, Laird AR, Bielfelt B, Griffin DD, Bányai K, Ramachandran et al. Serotype diversity and reassortment between human and animal rotavirus strains: Implications for Rotavirus Vaccine Programs. J Infect Dis. 2005 Sep;192 Suppl 1:S46-59.

6. Santos N, Hoshino Y, Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine. Rev. Med. Virol. 2005 Jan;15:29-56

7. Phan TG, Okitsu S, Maneekarn N, Ushijima H. Evidence of intragenic recombination in G1 rotavirus VP7 genes. J Virol. 2007 Sep;81(18):10188-94.

8. Vesikari T, Matson DO, Dennehy P, Damme PV, Santosham M, Rodriguez Z, et al. Safety and efficacy of a pentavalent human-bovine (WC3) reassortant rotavirus vaccine. N Engl J Med. 2006 Jan;354(1):23-33.

9. Ruiz-Palacios GM, Pérez-Schael I, Velázquez R, Abate H, Breuer T, Costa SA, et al. Safety and efficacy of an attenuated vaccine against severe rotavirus gastoenteritis. N Engl J Med. 2006 Jan;354(1):11-22.

10. Bernstein DI. Live Attenuated human rotavirus vaccine, Rotarix. Semin Pediatr Infect Dis. 2006 Oct;17(4):188-94.

11. Gurgel RQ, Cuevas LE, Vieira SC, Barros VC, Fontes PB, Salustino EF et al. Predominance of rotavirus P[4]G2 in a vaccinated population, Brazil. Emerg Infect Dis. 2007 Oct;13(10):1571-3.

12. Endara P, Trueba G, Solberg O, Bates SJ, Karina P, Cevallos W, et al. Symptomatic and subclinical infection with rotavirus P[8]G9, rural Ecuador. Emerg Infect Dis. 2007 Apr;13(4):574-9.

13. Das BK, Gentsch JR, Cicirello HG, Woods PA, Gupta A, Ramachandran M, et al. Characterization of rotavirus strains from newborns in New Delhi, India. J Clin Microbiol. 1994 Jul;32(7):1820-2.

14. Gentsch JR, Glass RI, Woods P, Gouvea V, Gorziglia M, Flores J, et al. Identification of group A rotavirus gene 4 types by polymerase chain reaction. J Clin Microbiol. 1992 Jun;30(6):1365-73.

15. Naranjo A, Cedeño C, Teran E, Castello A, and the CASERO Research Team. Prevalence of VP4 and VP7 genotypes of human rotavirus in Ecuadorian children with acute diarrhea. J Med Virol. 2008 Jun; 80(6):1106-11.

16. Khamrin P, Peerakome S, Tonusin S, Malasao R, Okitsu S, Mizuguchi M, et al. Changing pattern of rotavirus G genotype distribution in Chiang Mai, Thailand from 2002 to 2004: decline of G9 and reemergence of G1 and G2. J Med Virol. 2007 Nov;79(11):1775-82.

17. Rahman M, Sultana R, Ahmed G, Nahar S, Hassan Z, Saiada F, et al. Prevalence of G2P[4] and G12P[6] rotavirus, Bangladesh. Emerg Infect Dis. 2007 Jan;13(1):18-24.

18. Franco MA, Angel J, Greenberg HB. Immunity and correlates of protection for rotavirus vaccines. Vaccine. 2006 Apr 5; 24(15):2718-31.

19. Kobasa D., Takada A. Shinya K., Hatta M, Halfmann P., Theriault S., et al. Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus. Nature. 2004 Oct 7; 431(7017):703-7

20. Fischer TK, Steinsland H, Molbak K, Rui C, Gentsch JR, Valentiner-Branth P, *et al*. Genotype profiles of rotavirus Straits from children in a suburban community in Guinea-Bissau, Western Africa. J Clin Microbiol. 2000 Jan;38(1):264-7.

21. Fischer TK, Eugen-Olsen J, Pedersen AG, Molbak K, Böttiger B, Rostgaard K, *et al*. Characterization of rotavirus strains in a Danish population: High frequency of mixed infections and diversity within the VP4 gene of P[8] strains. J Clin Microbiol. 2005 Mar;43(3):1099-104.

22. Cunliffe NA, Gondwe JS, Graham SM, Thindwa BD, Dove W, Broadhead RL. Rotavirus strain diversity in Blantyre, Malawi, from 1997 to 1999. J Clin Microbiol. 2001 Mar;39(3):836-43.

23. Rahman M, Sultana R, Podder G, Faruque AS, Matthijnssens J, Zaman K, et al. Typing of human rotaviruses: nucleotide mismatches between the VP7 gene and primer are associated with genotyping failure. Virol J. 2005 Mar 24;2:24.

24. Parra GI, Espinola EE. Nucleotide mismatches between the VP7 gene and the primer are associated with genotyping failure of a specific lineage from G1 rotavirus strains. Virol J. 2006 May 25;3:35.

25. Iturriza-Gómara M, Green J, Brown DW, Desselberger U, Gray JJ. Diversity within the VP4 gene of rotavirus P[8] strains: implications for reverse transcription-PCR genotyping. J Clin Microbiol. 2000 Feb;38(2):898-901.

26. Fischer TK, Page NA, Griffin DD, Eugen-Olsen J, Pedersen AG, Valentiner-Branth P, et al. Characterization of incompletely typed rotavirus strains from Guinea-Bissau: identification of G8 and G9 types and a high frequency of mixed infections. Virology. 2003 Jun 20;311(1):125-33.

27. Fischer TK, Steinsland H, Valentiner-Branth P. Rotavirus particles can survive storage in ambient tropical temperatures for more than 2 months. J Clin Microbiol. 2002 Dec;40(12):4763-4.

28. Desde hoy se aplicará la vacuna del rotavirus. El Universo. 2007 Oct 18; Sect El gran Guayaquil.

29. Vacuna contra el rotavirus ya está en el Ecuador. Hoy. 2006 Jan 11; Sect. País.