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High Prevalence of P[8]G9 Rotaviruses in Remote Communities of Ecuadorian Coast

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HOJA DE APROBACIÓN DE TESIS

High Prevalence of P[8]G9 Rotaviruses in Remote Communities of Ecuadorian Coast

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Dedicatoria

Este trabajo, realizado con el mayor esfuerzo posible de manera que sea posible juzgarme por él, no por su presentación exterior sino por la calidad, honradez y exactitud con la que fue ejecutado, está dedicado a mi hijo Pablo Andrés. Debo decirle a él que es la perfección de las obras que nos toca realizar, uno de los factores de orden moral que contribuyen a la felicidad y a la tranquilidad de conciencia que resultan del deber bien cumplido.

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Resumen

Introducción: Epidemiológicamnte, los genotipos más comunes de rotavirus en el mundo son G1, G2, G3 y G4. Sin embargo, en la pasada década el aislamiento del genotipo G9 y su diseminación a través de varias regiones del mundo ha significado la reevaluación de las implicaciones epidemiológicas y sobre todo de medidas preventivas.

Métodos: Las muestras de heces de individuos pertenecientes a 22 comunidades rurales de la provincia de Esmeraldas fueron recolectadas entre agosto del 2003 hasta febrero del 2006. También se recolectaron muestras de heces de niños afectados con diarrea del Hospital Baca Ortiz durante marzo del año 2006. Las muestras de heces fueron genotipificadas por medio de la técnica de RT-PCR.

Resultados: De las comunidades rurales se recolectaron 1656 muestras de heces, de las cuales 411 fueron de casos de diarrea y 1245 fueron controles. En total 136 muestras fueron positivas para la detección del rotavirus, 96 (23,3%) pertenecientes a casos y 40 (3,2%) a controles. La genotipificación realizada por RT-PCR demostró una predominancia del genotipo P[8]G9. Las 29 muestras recolectadas del Hospital en Quito mostraron también la predominancia del mismo genotipo.

Conclusiones: La predominancia del genotipo P[8]G9 como la cepa dominante ha sido reportada en pocos países. El potencial de diseminación que ha demostrado la cepa P[8]G9 podría significar cambios en las formulaciones de las vacunas con el fin de que estas contengan a esta cepa emergente.

Abstract

Background: Next to the common G1-G4 human rotavirus genotypes, the newly emerging G9 genotype has been spreading throughout the world during the past decade carrying epidemiological and preventive implications.
Methods: Stool samples were collected in 22 remote communities along the northern coast of Ecuador between August 2003 and February 2006. Additionally, stool samples were collected from a hospital in Quito during March 2006. The genotype of the strains was assessed by multiplex RT-PCR.
Results: From a total of 1656 community stools samples (411 cases and 1245 controls), 96 cases (23,3%) and 40 controls (3,2%) were found positive for rotavirus. The genotype of the strains was assessed by multiplex RT-PCR and showed a predominance of genotype P[8]G9. The 29 samples obtained from a hospital in Quito showed a similar genotype distribution.
Conclusions: Few countries have reported the P[8]G9 rotavirus genotype as the dominant strain. The potential for the growing presence of this genotype may require changes to current vaccination programs to include coverage for this genotype.

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1. BACKGROUND.

Rotavirus is the single most important cause of acute gastroenteritis and death in infants and young children worldwide, causing an estimated 352,000-592,000 annual deaths in children less than five years of age [1]. Although the incidence of infection in children in developed and developing countries is similar, the outcome varies significantly. In countries classified by the World Bank as high-income, the risk of dying from rotavirus before the age of five is 1 in 48,680; the equivalent risk in low-income countries is 1 in 205 [2]. Besides the oral or intravenous rehydration treatment, the development of vaccines candidates, as preventive measure, has been going on since the early 1980s. The development of a vaccine can be reached with regional epidemiological and molecular approaches in different geographical zones due to the particular distribution of the genotypes of rotavirus strains.

Rotaviruses are classified as a genus in the *Reoviridae* family. The rotavirus genome is comprised of 11 double-stranded RNA segments; each segment encodes a unique structural or non-structural protein. Table 1 shows this proteins and their function.

Genomic segment	Protein	Localization	Function
1	VP1	Core	RNA polymerase
2	VP2	internal capside	RNA ligador
3	VP3	Core	Guanilyltransferasa
4	VP4	external capside	Infectivity
5	NSP1	Non-structural	RNA ligador
6	VP6	Midle Capside	Group Antigen
7	NSP3	Non-structural	RNA ligador
8	NSP2	Non-structural	RNA ligador
9	VP7	Capside externa	Neutralizing antgen
10	NSP4	Non-structural	Viral enterotoxin
11	NSP5	Non-structural	RNA ligador

Table 1. Genomic segments and proteins of rotavirus.

The viral particle is a wheel-like shape, non-envelope, triple-layered icosahedral virus. The inner most layer is formed by VP2 proteins, which is surrounded by a sheet of

VP6 proteins [1, 3, 4]. The outer layer contains two antigenic proteins: VP7 and VP4. The VP7 glycoprotein is the major neutralization antigen of rotavirus detected by hyper immune antiserum and has served as the basis for identification of serotype. It is located on the outer capside forming the smooth external surface of the outer shell, and constitutes 30% of the virion protein [5]. VP4 is a nonglycosylated protein present on the outer capside as a series of spikes with knoblike structure at the distal end and constitutes 1,5% of the viral proteins. Although is a minor component of the outer capside is the attachment protein, and protease treatment cleaves VP4 into VP5 and VP8, enhancing viral infectivity. However cleavage is not required for binding of virus to host cells [5]. VP7 and VP4 are referred to as the G protein (glycoprotein) and the P protein (protease-sensitive) respectively [3, 4].

To date 15 G-genotypes and 23 P-Genotypes have been identified [4]. The combination of these two outer proteins constitutes the viral genotype. Due to the segmented nature of the rotavirus genome, the genes for the external structural proteins may segregate independently during co-infections (genetic reassortment), increasing the genetic diversity of rotaviruses. A dual nomenclature referring to the G and P proteins is used to classify the viruses [1, 6].

Humans are infected by at least 10 G genotypes and 11 P genotypes. Data from 1994-2003 indicate that the four most prevalent combinations worldwide of G and P genotypes were: P[8]G1 (52%), P[4]G2 (11%), P[8]G4 (8 %), and P[8]G3 (3 %), which represented almost 74 % of the global isolates [3]. The prevalence of these four viral types was similar in Latin America (68%) during the same period [4, 7].

More recent data suggest that G9 type is gaining global importance in the last ten years [4, 8-12]. The P[8]G9 type, the most common combination, may

have resulted from a reassortment event between the most prevalent type P[8] and a strain carrying G9 [9].

Between 1990 and 2004, P[8]G9 rotaviruses caused less than 5% of rotavirus infections worldwide but 15% of infections in South America [3, 4], suggesting the earlier spreading of this genotype in the region.

Besides epidemiological importance, genotyping of circulating strains has an enormous relevance for vaccine development. The most efficacious vaccination protocols are those utilizing viral serotypes similar to those circulating in a given community (homotypic responses) [4, 13]. Vaccinations with serotypes distinct from those circulating (heterotypic), are less effective [12-14]. Therefore local determination of rotavirus genotypes is critical for prevention of disease.

Although rotaviral infections have been reported in Ecuador [15], to our knowledge, this is the first report in which circulating rotavirus strain are characterized on the molecular level in Ecuador.

The data presented here are unique in that they are community-based and include all symptomatic individuals as well as asymptomatic controls. This approach differs from most rotavirus genotyping studies, which focus on patients in a clinical setting. The present study thus describes the total illness rate associated with rotavirus infection from 22 remote, rural communities on the northern coast of Ecuador.

2. METHODS.

2.1. Study Population and Study Design.

As part of a larger case-control study, fecal samples from persons in 22 remote communities located in Esmeraldas, the northernmost province on the coast of Ecuador. Each of 21 small, rural communities was visited four times, each time for 15 days, from

August 2003 through February 2006. Fecal samples were also collected from the region's largest town, Borbón, for 15 days in July 2005. During each 15-day visit, health workers visited every household and interviewed residents to identify every case of diarrhea. For each identified case of diarrhea, three asymptomatic controls were selected, one from the case-patient's household and two randomly selected from the community. A total of 1656 stools samples were collected, 411 (25%) of which were from cases of diarrhea. In order to determine whether the results in these remote communities were representative of rotavirus infections in other Ecuadorian locations, 29 fecal samples from diarrheic rotavirus cases were obtained from the Hospital de Niños Baca Ortiz in Quito and also analyzed. Protocols were approved by the bioethics committee at the Universidad San Francisco de Quito and the Internal Review Board at University of California Berkeley, California, USA.

2.2. Rotavirus Detection.

All of the 1,656 fecal samples recovered (symptomatic and non-symptomatic) were analyzed by a commercial immunochromatographic test (RIDA Quick Rotavirus, R-Biopharm AG). Positive samples were stored in liquid nitrogen before RT-PCR analysis.

2.3. Rotaviral multiplex RT-PCR.

The double-stranded rotaviral RNA was extracted from the stools by the Trizol® Method (Invitrogen), or by the commercially available UltraClean® RNA Tissue Isolation Kit (MoBio Laboratories Inc). RNA was stored at -80˚C until further use. For sequencing purposes, samples were transferred directly onto chromatography paper strips treated with SDS-EDTA, dried overnight at room temperature, and sent to Belgium by standard postal service [16]. A semi-nested multiplex RT-PCR was carried out for G- and P-genotyping based on a protocol provided by Jon Gentsch and described elsewhere [17]. Briefly, primers *9con1* and *9con2* [18] were used for the first amplification of the VP7 gene sequence (G-typing) and primers *con3* and *con2* [19] were used for the partial

amplification of the VP4 gene (P-typing). For the second, multiplex RT-PCR, the following G-genotype specific primers were used: *9T-1*, *9T-2*, *9T-3P*, *9T-4*, and *9T-9B* [18], resulting in type-specific PCR products sizes. For the equivalent P-typing, the following specific primers were used: *1T-1*, *2T-1*, *3T-1*, *4T-1*, *5T-1*, and *ND2* [19]. The sequences are described in Table 2.

VP7 (G) Typing		**VP4 (P) Typing**	
Primer	**Sequence (5' to 3')**	**Primer**	**Sequence (5' to 3')**
1st amp consensus primers		**1st amp consensus primers**	
9con1	tag ctc ctt tta atg tat gg	con3	tgg ctt cgc tca ttt ata gac a
9con2	gta taa aat act tgc cac ca	con2	att tcg gac cat tta taa cc
2nd amp primers		**2nd amp primers**	
9T-1	tct tgt caa agc aaa taa tg	1T-1	tct act tgg ata acg tgc
9T-2	gtt aga aat gat tct cca ct	2T-1	cta ttg tta gag gtt aga gtc
9T-3P	gtc cag ttg cag tgt agc	3T-1	tgt tga tta gtt gga ttc aa
9T-4	ggg tcg atg gaa aat tct	4T-1	tga gac atg caa ttg gac
9T-9B	tat aaa gtc cat tgc ac	5T-1	atc ata gtt agt agt cgg
		ND2	agc gaa ctc acc aat ctg

Tabla 2. Primers used in multiplex RT-PCR.

Five ul of viral RNA was denatured for 5 min at 97˚C. Retrotranscription and the first amplification were carried out using a SuperScript III ™ RT/Platinum Taq polimerase

kit (Invitrogen). Primers were used at 200 nM each, and the 1X buffer provided by the manufacturer contained 1.6 mM $MgSO_4$ and 200 μM of dNTPs. The retrotranscription was carried out at 42˚C for 45 min and stopped at 96˚C for 2 min. The first amplification consisted of 30 cycles at 94˚C for 30s, 50˚C for 30s, and 72˚C for 60s. The second amplification was carried out using PuReTaq Ready-To-Go™ PCR beads (Amersham Biosciences) and primers at a final concentration of 400 nM. The cycling parameters were 30 cycles at 94˚C for 30s, 42˚C for 30s and 72˚C for 60s, and a final extension at 72˚C for 1 min.

The amplification products (10 ul) were analyzed by electrophoresis running, using a 6X gel loading buffer in a 1.8% agarose gel.

2.4. Nucleotide Sequencing.

A total of 22 PCR products that were identified as P[8]G9 from rural samples were purified with the QIAquick® PCR purification kit (Qiagen/Westburg), and sequenced with the ABI PRISM® BigDye Terminator Cycle Sequencing Reaction kit (Applied Biosystems) on an ABI PRISM™ 3100 automated sequencer (Applied Biosystems). For sequencing purposes, *Beg9* and *End9* primers were used for the VP7 gene and *1-17F* and *Con2* for the VP4 gene. The sequencing reaction conditions were: 25 cycles at 94°C for 15s, 50°C for 15s, and 72°C for 4 min), and a final extension of 72°C for 7 min.

2.5. Sequences alignment and phylogenetic analysis.

In order to stablish a phylogenetic comparison nineteen partial VP7 DNA sequences from rural communities were aligned with 3 references strains obtained from Gene Bank using the CLUSTAL W algorithm. A phylogenetic tree was constructed using a Neighbor-joining method, and bootstrap support was calculated using 100 replicates in MEGA 3.1 program.

2.6. Nucleotide Sequence Accession Numbers.

Sequence data for VP7 gene from the 22 G9 community isolates were deposited in GenBank under accession numbers DQ848566-DQ848587.

3. RESULTS.

Of 1656 fecal samples from remote communities analyzed for rotavirus, 136 (8.2%) were determined to be positive by the commercial immunodetection test. Of these positive samples, 96 (70,6%) were from patients with diarrhea and 40 (29,4%) were from asymptomatic controls. Diarrhea was significantly associated with being infected with rotavirus (OR = 8.0, 95% CI: 5.3, 12.3). Interestingly, no significant difference in rotavirus incidence was observed between the different age groups (Table 3). The samples recovered from Quito all came from children under 5 years old and all of them had diarrhea symptoms.

	Age group				
	< 1y +/N (%)	1-4 y +/N (%)	5-10 y +/N (%)	>10 y +/N (%)	Total +/N (%)
Cases	18/62 (29.0%)	33/181 (18.2%)	7/36 (19.4%)	20/77 (26.0%)	78/356 (21.9%)
Controls	0/25 (0%)	6/143 (4.2%)	10/238 (4.2%)	24/778 (3.1%)	40/1184 (3.4%)

Table 3. Age Distribution of Rotaviral Positive Cases (those with diarrhea) and Controls (those without diarrhea) in Rural Communities of Esmeraldas. The number of positive (+) and tested cases (N) is reported.

The multiplex RT-PCR analysis yielded successful PCR amplification in 39 of the 136 (29%) samples. Thirty four samples (87%) of the 39 positive reactions, produced a PCR product with a size corresponding to the G9 genotype, and 31 samples (80%) produced a PCR product corresponding to P[8]. (Figure 1A and 1B).

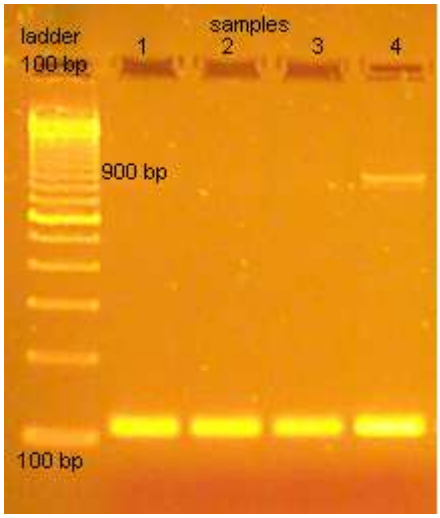

Figure 1A. Electrophoresis gel shows a typical 110 bp band of G9 genotype in lanes1-4. Additionally one band of 900 bp in lane 4 correspondent to the first amplification product.

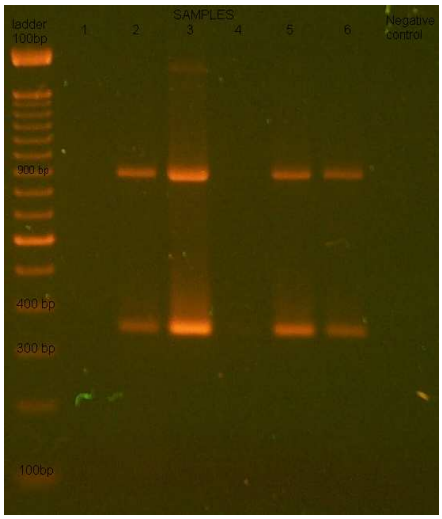

Figure 1B. Electrophoresis gel shows a 330 bp band correspondent to P[8] genotype in lanes 2, 3 and 5, 6. No amplification was achieved in lanes 1 and 4. Negative control was included in line 7.
First amplification products of 880 bp are shown in the same lanes where P[8] bands were detected.

The P[8]G9 combination was found in 29 samples (74%) of the samples. A small proportion of the samples produced patterns corresponding to P[6]G1 (8%) and P[6]G9 (3%) (Table 4). The identity of the P[8]G9 bands was confirmed by DNA sequencing.

Location	Genotypes determined by multiplex RT-PCR								
	P[6]G1	P[6]G9	P[8]G9	P[8]G_{int}	P[int]G9	P[4]/P[8] G2	P[4]/P[8] G2/G9	P[8] G2/G9	P[6]/P[8] G9
Esmeraldas	3	1	29	2	4	0	0	0	0
Quito	1	0	21	0	1	1	2	1	1

Table 4. Distribution of G and P types in remote communities in Esmeraldas and Quito. Indeterminant G and P typing is designated as G_{int} and P[int] respectively.

To determine whether the serotypes in remote communities corresponded to strains circulating in other places of Ecuador, we analyzed 29 rotavirus positive samples from Hospital de Niños Baca Ortiz in Quito. There were 28 successful amplifications in the 29 samples. The majority of the samples 26 of 29 corresponded to G9 (93%) and 25 of 28 (89%) to P[8] and combination P[8]G9 was found in 24 of 28 (86%) samples.. Only one sample showed a pattern corresponding to the P[6]G1 genotype (Table 4). Additionally we found electrophoretic evidence for 5 mixed infections in which the G2 and G9 genotypes and the P[4], P[6] and P[8] genotypes were involved in several combinations (Table 4 and Figure 2A and 2B).

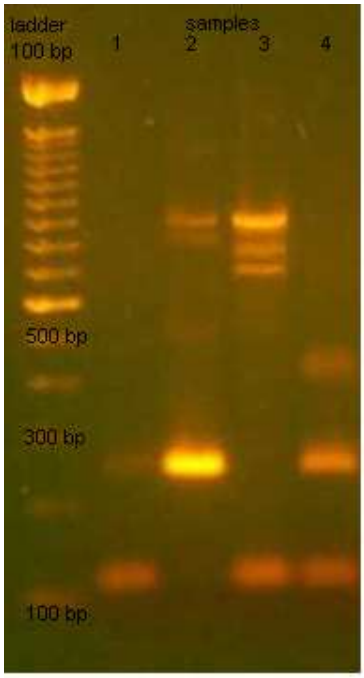

Figure 2A. Gel electrophoresis for G typing showing in lane 4 three bands: 110 bp, 250 bp, and 460 bp in concordance with mixed infection with G9, G2, and G3 strains. Lanes 1 and 3 with 110 bp band (G9 strain) and lane 2 with 250 bp band corresponded to G2 strain.

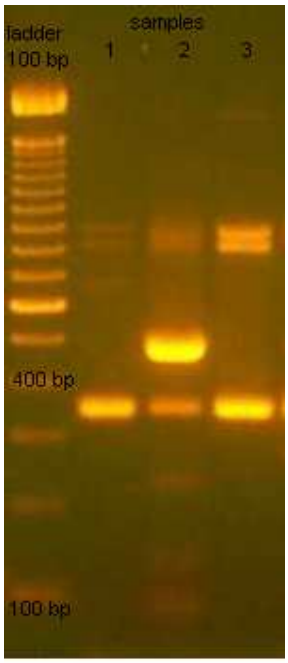

Figure 2B. Gel electrophoresis for P typing. Showing a mixed infection in lane 2 with bands of 240 bp (faint band), 330 bp and 460 bp, in concordance with P[6], P[8] and P[4] strains.

From the 29 P[8]G9 rural community samples, 22 were sent for sequencing at the University of Leuven, Belgium. About 750 bp of high-quality nucleotide sequence data for the VP7 gene were obtained from each sample. The 22 P[8]G9 samples were remarkably homogenous at the sequence level, with only three single nucleotide polymorphisms found present in the 22 sequences.

Phylogenetic analysis of the sequences (Figure 3) showed that the Ecuadorian sequences grouped together monophyletically and are more related with recently isolated G9 rotavirus sequences.

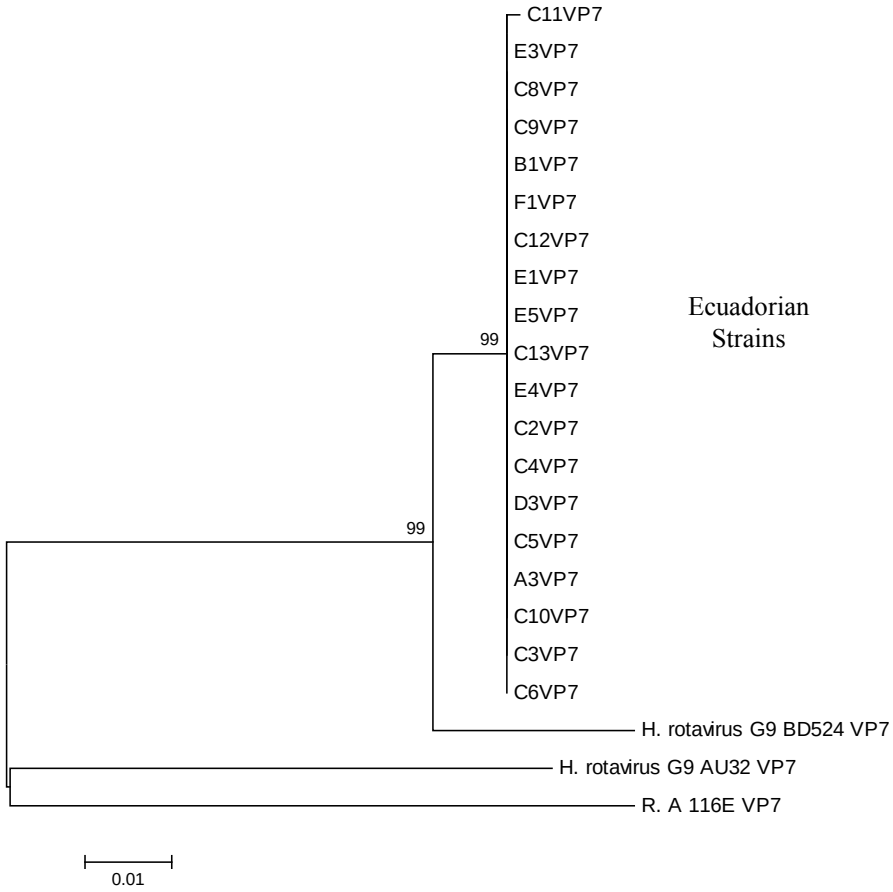

Figure 3. Phylogenetic tree of VP7 sequences of 19 rural samples from Ecuador and three reference strains: BD524, Accession number AJ250543 (lineage III); E116, Accession number L14072 (lineageI) and AU32, Accession number AB045372 (lineage II). using Bootstrap test by Neighbor-Joining method. Mega3.1 Program.

4. DISCUSSION.

In this first description of rotavirus genotyping in Ecuador, we report a high rate of infection with rotavirus G9 (87%) in two geographically distinct regions within Ecuador, a remote coastal rain forest and an urban Andean Hospital. Although G9 has been considered uncommon, recent reports have described it as increasingly important [3, 4, 8-12, 20-22]. These results support the observation that the G9 genotype, particularly P[8]G9, is spreading throughout Latin America. To our knowledge, however, only two studies have reported G9 isolation rates as

high as the present study: in Salvador, Brazil, the rate was 75-90% between 1999-2002 [12], and in Chiang Mai, Thailand, it was 92% in 2000-01 [23].

The lack of differences between age groups infected with rotavirus may be due to that the present study appears to be one of the few community-based descriptions of rotavirus infection. Symptomatic individuals were actively identified in the community, recruited into the study, and matched with three asymptomatic controls each. This approach presents a more complete picture of rotavirus infection in rural communities than would be possible with the clinical sampling used in most previous studies that presumably focused in on more urban environments. The high rate of rotavirus infections among symptomatic persons >40 years old of age may be due to this age group's lack of exposure to the emerging rotavirus genotype and is a observation that might have been missed in a purely clinical study.

Additional evidence that G9 rotavirus is spreading throughout Latin America comes from comparing our nucleotide sequences to other sequences reported in GenBank. The sequences from the current study cluster into a large clade, which includes most of the recently isolated G9 rotavirus reported in the literature. This "emergent clade" is relatively homogenous: most isolates within the clade have ≤1% sequence divergence.

Phylogenetic analysis showed that all the Ecuadorian samples are more closely related with BD524 strain (lineage III) isolated in Bangladesh in 1996, than the older viral isolations E116 strain (lineage I) and AU32 strain (lineage II) which were isolated in India 1985 and Japan 1986 respectively [24, 25] (Figure 3). This data could indicate that Ecuadorian strains belong to a novel strain that differs from the earliest G9 strains, and have acquired evolutive characteristics to spreading and establish itself like a fifth more common rotavirus strain worldwide.

A potential source of bias in this study comes from the incomplete typing of the putatively rotavirus-positive specimens. These incomplete samples, which were positive by immunochromatographic tests, may be the result of inappropriate handling or storage of some fecal samples, which can be complicated in remote community studies such as this. We think that typing failure was because of RNA degradation.

The increasing prevalence of G9 rotavirus is particularly relevant given that many countries, including Ecuador, have approved the use of two rotavirus vaccines. Rotarix® contains an attenuated P[8]G1 human rotavirus (HRV) [14] and the Rota-Teq® a live pentavalent human-bovine (WC3 strain) reassortant rotavirus vaccine containing human serotypes G1, G2, G3, G4, and P[8] [26]. Despite the wide distribution of G9 during the last 9 years [3, 4, 8-12, 27], and growing evidence of this strain in Latin America, both vaccine formulations currently available do not include serotype G9 antigen [14, 26]. Studies have shown that some vaccines which do not contain G9 antigen may still be capable of eliciting protective immunity against the G9 serotype [14]. This immunity is most likely attributable to the fact that G9 is mostly found in combination with P[8], which is included in both vaccines. However, cross immunity may not be universal as has been seen with type P[4]G2 [3, 4, 14]. Continual surveillance of circulating types, therefore, should be carried out before to the introduction and during the implementation of rotavirus vaccination programs.

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