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Molecular characterization of group A rotaviruses circulating in Senegal over a 7-year period (2005-2011)

Diop, A.[1], Dia, M. L.[1]*, Sonko, M. A.[1], Diop, D.[1], Kâ, R.[1], Sow, A. I.[1], Bâ, M.[2] and Cissé, M. F.[1]

[1]Department of Bacteriology and Virology, Faculty of Medicine. Dakar, Senegal.
[2]Albert Royer Children's Hospital, Dakar, Senegal.

Group A rotaviruses are characterized by large genomic diversity that is typically studied by genotyping of the VP7 glycoprotein, which defines G-type, and the protease-sensitive VP4, which defines P-type. The aim of this study was to examine the genotypes of group A rotavirus strains circulating in Senegal over a 7-year period between 2005 and 2011. ELISA positive stool specimens were analyzed by PAGE and were genotyped by multiplex hemi-nested RT-PCR. Data were analyzed with Epi Info 7 software. Rotavirus was found in 222 samples of stools of which 32 (14.41 %) were positive on PAGE. Five electropherotype patterns were identified, three long (L1, L2 or L3) and two short (S1 or S2). Twenty-three (23) samples had a long electropherotype and nine had a short electropherotype. One hundred strains (45.04%) were analyzed by VP7 genotyping, which identified five different genotypes: G1, G2, G3, G8 and G9. Ninety-three (93) samples (41.89%) were analyzed by VP4 genotyping which showed four different genotypes: P[4], P[6], P[8] and P[11] and mixed genotypes (1.8%). Seventy seven (34.68%) samples were genotyped for both VP7 and VP4. The G1P[8] strain was the most predominant strain followed by the G9P[8] strain. Rotavirus strains circulating in Senegal are genetically diverse. Genotypes G1P[8] and G9P[8] are the predominant strains. We also found mixed infections that could favor the emergence of new viral strains. Thus, regular monitoring of genotypes circulating in the country is required.

Key words: Rotavirus, diarrhea, genotypes, children, Senegal.

INTRODUCTION

Human group A rotaviruses (RVA) are ubiquitous and are the leading cause of diarrhea in children under five years old. In Senegal, rotavirus is the principal cause of pediatric diarrhea during the dry season (Sambe-Ba et al., 2013). The virus appears as a wheel-shaped particle on electron microscopy. These non-enveloped viruses have an icosahedral capsid composed of a triple layer of proteins (VP2, inner layer; VP6, middle layer; VP7+VP4, outer layer) and a genome made of 11 segments of double-stranded RNA (dsRNA) (Estes and Greenberg, 2013). The viral RNA migration pattern obtained by polyacrylamide gel electrophoresis (electropherotype) is used as a marker in epidemiology, with differences in migration of one or more RNA segments distinguishing

rotavirus strains (Estes and Greenberg, 2013). However, the method of choice for RVA classification today is genotyping by reverse transcription-polymerase chain reaction [RT-PCR]' for classification of RVAs into groups/species (Matthijnssens et al., 2012). The three main antigenic proteins of the virus can be used to classify rotavirus into seven groups labeled A to G (VP6), and into G (VP7) and P (VP4) genotypes (Estes and Greenberg, 2013). At least 27 G-types and 37 P-types have been characterized so far (Trojnar et al., 2013), and various combinations of G- and P-types exist (Estes and Greenberg, 2013; Rahman et al., 2005). The diversity of strains circulating in different regions is important for vaccine development and clinical assessment (Gentsch et al., 2005; Santos and Hoshino, 2005).

Although the G9 G-type is considered one of the major human genotype of RVA worldwide (Ianiro et al., 2013), G8, G9 and G12 genotypes, which are not included in the vaccines, have appeared more regularly over the past few years (Matthijnssens et al., 2010). The emergence of these new genotypes reveals the importance of monitoring rotavirus infections before and after the introduction of vaccines. The major aim of this study was to study the genotypes of RVA strains circulating in Senegal over a 7-year period, between 2005 and 2011.

MATERIALS AND METHODS

Samples collection

This was a prospective study carried out between January 1st, 2005 and December 31st, 2011. Stool samples were collected from children with diarrhea, who were treated as inpatients or outpatients at five hospitals: three in Dakar (Albert Royer Children's Hospital, Pediatric Institute of Pikine and Abass Ndao Hospital) and one in Saint-Louis (263 km from Dakar) and one in Diourbel (146 km from Dakar). Most children were under 5 years old.

Samples were collected in an airtight container and taken immediately to the laboratory. Containers were placed in an icebox in case transportation was delayed.

Detection of RVA

A commercial ELISA kit (IDEIA Rotavirus, DAKO Ltd, Glostrup, Denmark) was used to detect RVA. This kit contains a polyclonal antibody detecting proteins specific to RVA, notably the internal capsid protein VP6. It is a sandwich-type ELISA test that can be read either with the naked eye or through a spectrophotometer. Positive samples were frozen at -80°C for PAGE analysis and genotyping at one of three reference laboratories: the Pasteur Institute virology laboratory in Dakar and two WHO collaborative centers, the National Health Laboratory Service (NHLS) in South Africa and the Medical Research Council (MRC) in The Gambia. Statistical analysis was carried out with Epi Info. 7 software.

Extraction of viral RNA with TRI-Reagent1

Viral RNA was extracted with TRI-Reagent1 (Sigma) according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi (1987).

Separation of viral genome by Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis was performed on two polyacrylamide gels of different concentrations: - a 10 % gel, which enables most of the viral genome to be separated into various segments; - a 3 % gel, which facilitates the separation of each segment. Extracted RNA was mixed with bromophenol blue and glycerol before loading onto the migration gel.

The migration was performed in Tris-glycine buffer for 22 h at 100 V. Bands were detected by silver staining and the gel was subsequently placed between two cellophane sheets and put into a gel dryer for 90 min.

Molecular typing of rotavirus strains

Denaturation of double-strand RNA (dsRNA) segments and annealing of external primers on single-strand RNA

Five (5) µl of dsRNA extract was added to 1 mmol of each primer (sense and antisense) with 3 µl of DEPC-treated water in an Eppendorf tube. The mixture was put into a thermocycler at 97°C for 5 min then immediately cooled on ice.

Reverse transcription

A total of 40 µl of a reaction mixture containing 24 µl of DEPC-treated water, 2 mmol of dNTPs, 5 µl of reverse transcriptase buffer, 2 mmol of Dithiothreitol (DTT), 4 mmol of $MgCl_2$ and 0.05 U of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega1) was added to the denatured RNA. Tubes were then put into the thermocycler at 42.8°C for 45 min.

First PCR

PCR was carried out with all of the RT product. A total of 25.7 µl of sterile distilled water, 20 µl of Taq polymerase buffer, 10.02 mmol of dNTPs, 0.25 mmol of $MgCl_2$, and 1.5 U of Taq polymerase (Go-Taq, Promega1) were added to each tube, and PCR was performed with 30 cycles of 1 mindenaturation at 95.8°C, 1 minute hybridization at 42°C and 1 minute elongation at 72°C. These cycles were preceded by a denaturation step of 5 min at 95.8°C, and then followed by an elongation step for 7 min at 72°C.

Hemi-nested multiplex PCR

The PCR product was subjected to a second amplification by hemi-nested multiplex PCR with internal primers (Table 1) (Iturriza-Gomara et al., 2004). The quantity of DNA used at this step depended on the band intensity resulting from the first amplification, and ranged from 0.5 µl at high band intensity up to 6 µl at low band intensity. Afterwards, 0.5 mmol of $MgCl_2$, 0.2 mmol of dNTPs, 0.2 mmol of each primer, 10 µl of PCR buffer, 1.5 U of Taq polymerase and enough sterile water to obtain a final volume of 50 µl were added to the first PCR product. The mixture was prepared on ice, and then placed into the thermocycler for a second round of amplification similar to the protocol described above.

Detection of PCR products

The resulting PCR products were subjected to electrophoresis in a 2% agarose gel containing 0.0015% of ethidium bromide. A molecular weight marker (100 bp ladder; Promega1) was also

Table 1. G and P consensus and type-specific recommended primers.

Primer	Sequences (5'–3')	nt Position	Amplicon (bp)
G-typing (a)			
1st round			
VP7-F	ATGTATGGTATTGAATATACCAC	(nt 51–71)	881
VP7-R	AACTTGCCACCATTTTTTCC	(nt 914–932)	
2nd round			
G1	CAAGTACTCAAATCAATGATGG	(nt 314–335)	618
G2	CAATGATATTAACACATTTTCTGTG	(nt 411–435)	521
G3	ACGAACTCAACACGAGAGG	(nt 250–269)	682
G4	CGTTTCTGGTGAGGAGTTG	(nt 480–499)	452
G8	GTCACACCATTTGTAAATTCG	(nt 178–198)	754
G9	CTTGATGTGACTAY[a]AAATAC	(nt 757–776)	179
G10	ATGTCAGACTACAR[b]ATACTGG	(nt 666–687)	266
VP7-R	As above		
P-typing (b)			
1st round			
Con-3	TGGCTTCGCCATTTTATAGACA	(nt 11–32)	876
Con-2	ATTTCGGACCATTTATAACC	(nt 868–887)	
2nd round			
P[4]	CTATTGTTAGAGGTTAGAGTC	(nt 474–494)	483
P[6]	TGTTGATTAGTTGGATTCAA	(nt 259–278)	267
P[8]	TCTACTGGR[b]TTR[b]ACN[c]TGC	(nt 339–356)	345
P[9]	TGAGACATGCAATTGGAC	(nt 385–402)	391
P[10]	ATCATAGTTAGTAGTCGG	(nt 575–594)	583
P[11]	GTAAACATCCAGAATGTG	(nt 305–323)	312
Con-3	As above		

[a]Y= C or T; [b]R= A or G; [c]N= A, G, C or T.

included. The migration buffer was Tris–Borate–EDTA (composed of 0.45 M Tris-Borate, 0.01 M EDTA, pH 8.3). DNA bands were visualized by ultraviolet (UV) light.

RESULTS

Detection rate and distribution of rotavirus according to year

Rotaviruses were found in 222 stool samples. The distribution of the positive samples according to year is showed in Table 2.

Electrophoretic migration profiles of RVA found between 2005 and 2011

Among the 222 positive samples, 32 (14.41 %) were positive on PAGE. Five different electropherotypes were found during this six year period: 23 samples had a 'long' electropherotype (L1, L2 or L3) and 9 had a 'short' electropherotype (S1 or S2) (Figure 1).

Composition of VP7 genotypes

Among the 222 positive stools, 100 (45.04 %) were typed for VP7, which revealed five different genotypes: G1, G2, G3, G8 and G9. In 2005, G1 and G2 genotypes were predominant whereas most genotypes found in 2011 were G1 or G9 (Figure 2).
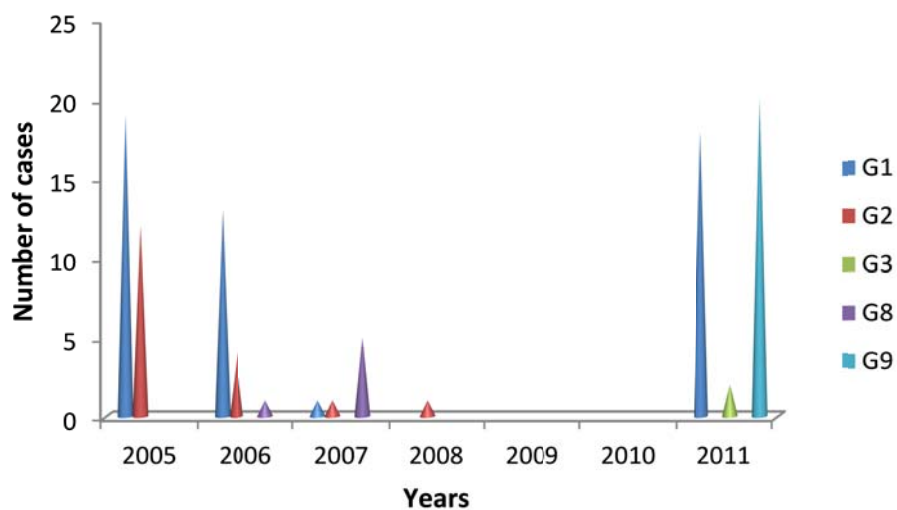
Composition of VP4 genotypes

Ninety-three (41.89 %) strains were typed for VP4, which showed four different genotypes: P[4], P[6], P[8] and P[11], and four mixed genotypes P[8]+P[6] (1.8%). Genotypes P[8] and P[6] were the predominant ones (Figure 3).

VP7/VP4 associations

Seventy-seven (34.68%) samples were typed for both VP7 and VP4, which identified several associations between genotypes (Table 2). The G1P[8] strain appears

Table 2. Evolution of VP4/VP7genotypes according to years.

Genotype	2005	2006	2007	2008	2009	2010	2011	Total
G1P6	2	6				1	2	11
G1P8	10	7					9	26
G1P11	1							1
G2P4	5			1				6
G2P6	3	5						8
G2P8	1							1
G8P6		1	3					4
G9P6		1				1	4	6
G9P8							14	14
GP typed	22	20	3	1	0	2	29	77
Out of	39	29	22	82	0	5	45	222
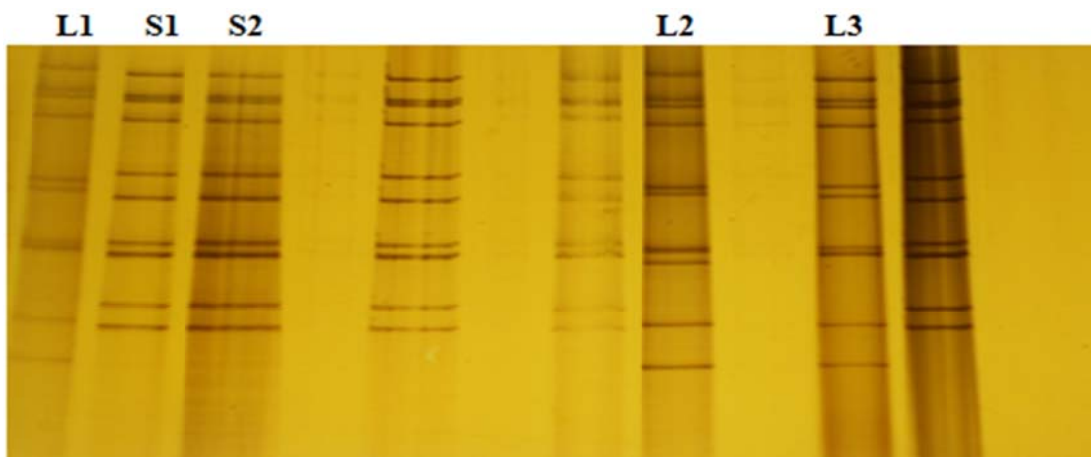
Figure 1. Electrophoretic profile with Polyacrylamide gel electrophoresis (PAGE). The polyacrylamide gel was visualized after silver staining. The RNA segments show the classic group A rotavirus distribution of bands. Three long electropherotypes (L1, L2 or L3) and two short (S1 or S2) are represented.

Figure 2. Composition of VP7 genotypes during the study period.
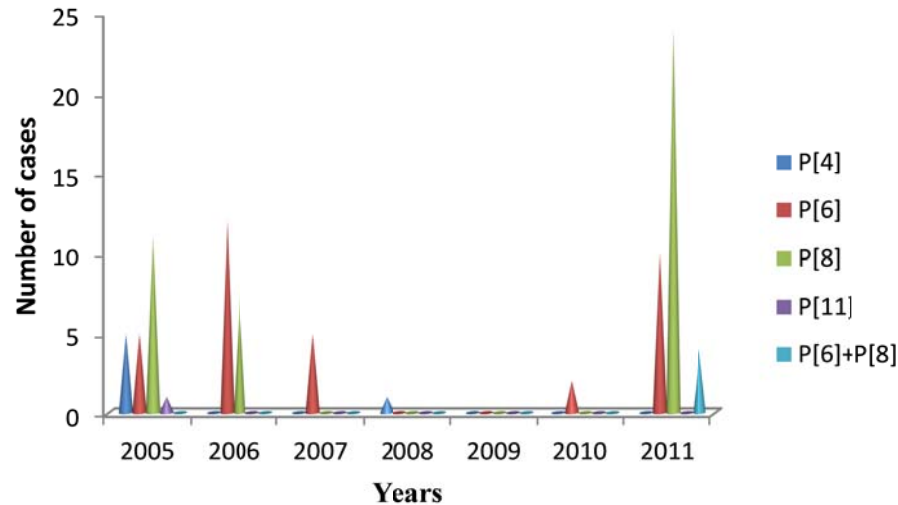
Figure 3. Composition of VP4 genotypes during the study period.

to be the predominant strain of RVA in Senegal (26 strains), followed by the G9P[8] strain (14 strains). This analysis also identified an unusual association between G2 and P[8].

DISCUSSION

Polyacrylamide gel electrophoresis

This is the first study of its kind to report the strains of RVA circulating in Senegal. We found that rotaviruses circulating during a 7-year study period were charac-terized by five different electrophoretic migration profiles; three long electropherotypes and two short electrophero-types. Long electropherotypes were more common than short electropherotypes in Senegal during the study period (n=23 *versus* n=9, respectively), consistent with a report from Tunisia (Chouikha et al., 2011). The co-circulation of many electrophoretic migration profiles has been described by other studies (Dzikwi et al., 2008), and is a characteristic of the epidemiology of rotavirus infections.

Composition of VP7 genotypes during the study period

Our study shows that the composition of circulating rotavirus G genotypes has changed throughout the years in Senegal. In 2005, the G1 genotype was predominant. The G3 genotype, which was absent in Senegal between 2005 and 2010, appeared in our study in 2011. The G2 genotype was present between 2005 and 2008. This genotype is the predominant G genotype in Tunisia, accounting for 52% of the strains found in this country

(Chouikha et al., 2011). It was also highly prevalent in Africa from 2007 through 2011, accounting for 16, 8% (Seheri et al., 2014).

The G8 genotype emerged in 2006 but disappeared quickly afterwards in 2007. We did not find the G4 genotype in our study, despite the fact that the G2, G3 and G4 genotypes are the most frequent genotypes worldwide after the G1 genotype and are circulating in variable proportions across many regions (Iturriza-Gomara et al., 2000).

The G9 genotype appeared in 2011 in our study. This genotype have been detected sporadically and in localized outbreaks in various African countries, including South Africa, Botswana, Malawi, Kenya, Cameroon, Nigeria, Ghana, Guinea-Bissau, Libya, and Mauritius (Page et al., 2010). Moreover, the G9 genotypes are "humanized" since the early 2000s, before they were a typical porcine RVA VP7 genotype. Today they are still one major VP7 genotype of RVA infecting pigs (Donato et al., 2012; Theuns et al., 2015).

Composition of VP4 genotypes during the study period

In 2005, the P[4], P[6] and P[8] genotypes were co-circulating in Senegal. These three genotypes are the most frequent VP4 genotypes worldwide (Iturriza-Gomara et al., 2011).
The P[8] genotype was the predominant form in 2005. In the following year, the P[6] genotype was the most abundant type, but was superseded by the P[8] genotype in 2011. The P[8] genotype is the most common geno-type worldwide: it accounts for 97.4% of P genotypes in Japan (Phan et al., 2007), 83.7% in Tanzania (Moyo et al., 2007) and 31% in India (Jain et al., 2001).

The P[11] genotype, which is rarely found in other countries, was isolated in our study in 2005. This genotype is currently emerging in several regions of the world (Esona et al., 2010). The prevalence of this genotype has been reported as 3% (Jain et al., 2001) and 8.5% (Banerjee et al., 2006) in studies conducted in India. It has also been described in Indonesia with a prevalence of 0.5% (Putnam et al., 2007).

Four samples had a mixed profile (P[8]+P[6]), which have been previously reported in several studies (Mwenda et al., 2010; Serravalle et al., 2007). Mixed profiles are particularly frequent during epidemics, because they are derived from genome assortment that occurs during mixed infections in the same child (Martella et al., 2010; Matthijnssens and Van Ranst, 2012).

Association between VP7/VP4 genotypes

The G1P[8] strain was predominant in Senegal between 2005 and 2011, consistent with the findings of other authors (Banyai et al., 2009). This association is one of the most frequent worldwide (Da Silva et al., 2015). However, the abundance of this strain declined in the following years and was replaced by an unusual association between G9 and P[8] in 2011. We also noted the emergence of another unusual combination between G8 and P[6] in 2006. However, the most frequently found associations involve stable combinations of genotypes with strong affinities for each other. For example, the P[4] genotype is often linked to the G2 genotype, whereas the P[8] genotype is most frequently associated with the G1, G3 and G4 genotypes (Abdel-Haq et al., 2003).

We found rotavirus strains with uncommon VP7/VP4 associations such as G2P[8]. Such associations have been previously reported in other studies, showing the variability of rotaviruses and re-assortment within rotavirus strains (Esona et al., 2010). Indeed, our data provide evidence of likely *in vivo* reassortment, for G/P constellations G1P[6], G1P[11], G2P[6], G2P[8], G9P[6], that is for 27/77 (35.1%) of fully G/P typed isolates. This is a high number which is not untypical in RVA strains collections from Africa (Iturriza-Gomara et al., 2001; Iturriza-Gomara et al., 2011). Furthermore, there may be RVA isolates carrying a G or P protein which is not covered by the primers used and therefore not genotyped. This is a frequent finding in African RVA strain (Seheri et al., 2014).

Few data about the P[11] genotype are available in literature (Matthijnssens et al., 2010). In our study, this genotype was associated with the G1 genotype, which is an unusual association. Indeed, a study carried out in India showed that P[11] genotypes are always associated with G10 genotypes (Jain et al., 2001). However, a Tunisian study detected the same G1P[11] association (Chouikha et al., 2011). We found the G9 genotype in association with the P[8] and P[6] genotypes. This geno-type is also associated with P[8] and P[4] in Cameroon

(Esona et al., 2010). Indeed, the G9 genotype has been reported to be in association with these VP4 genotypes, as well as the P[4] and P[19] genotypes. However, the G9 genotype is most frequently associated with P[8] (Zhou et al., 2001).

Overall, 1.8% of infections were mixed infections. This percentage is lower than that reported in other countries, including Brazil (29%) (Serravalle et al., 2007) and India (21%) (Jain et al., 2001). The high frequency of mixed infections in these countries facilitates the emergence of new strains (Fischer et al., 2005).

Conclusion

Our findings reveal a high diversity of rotavirus strains in Senegal during a 7-year period. Among these strains, the genotypes G1P[8] and G9P[8] were predominant. We also recorded mixed infections, which could favor the emergence of new strains, demonstrating the need for regular monitoring of genotypes circulating in the country. The coverage of both, sample collection and RVA genotyping has been very uneven over the years and this study is the start of more comprehensive work to be undertaken in Senegal.

Conflict of interests

The authors did not declare any conflict of interest.

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