

Characterization of incompletely typed rotavirus strains from Guinea-Bissau: identification of G8 and G9 types and a high frequency of mixed infections

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Abstract

Among 167 rotavirus specimens collected from young children in a suburban area of Bissau, Guinea-Bissau, from 1996 to 1998, most identifiable strains belonged to the uncommon P[6], G2 type and approximately 50% remained incompletely typed. In the present study, 76 such strains were further characterized. Due to interprimer interaction during the standard multiplex PCR approach, modifications of this procedure were implemented. The modified analyses revealed a high frequency of G2, G8, and G9 genotypes, often combined with P[4] and/or P[6]. The Guinean G8 and G9 strains were 97 and 98%, respectively, identical to other African G8 and G9 strains. Multiple G and/or P types were identified at a high frequency (59%), including two previously undescribed mixed infections, P[4]P[6], G2G8 and P[4]P[6], G2G9. These mixed infections most likely represent naturally occurring reassortance of rotavirus strains. Detection of such strains among the previously incompletely typed strains indicates a potential underestimation of mixed infections, if only a standard multiplex PCR procedure is followed. Furthermore cross-priming of the G3 primer with the G8 primer binding site and silent mutations at the P[4] and P[6] primer binding sites were detected. These findings highlight the need for regular evaluation of the multiplex primer PCR method and typing primers. The high frequency of uncommon as well as reassortant rotavirus strains in countries where rotavirus is an important cause of child mortality underscores the need for extensive strain surveillance as a basis to develop appropriate rotavirus vaccine candidates.
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Introduction

Human group A rotaviruses are the most frequently identified etiologic agent in children hospitalized with acute, severe, dehydrating diarrhea worldwide (Kapikian et al., 2001).

It is estimated that 400,000 to 600,000 children, most of whom live in developing countries, die from rotavirus infections each year (Miller and McCann, 2000). Children in sub-Saharan Africa account for 25 to 35% of these deaths (Molbak et al., 2000). Severe rotavirus diarrhea can be effectively prevented in young children by vaccination. Therefore, it was a major setback when the first licensed human rotavirus vaccine, the tetravalent Rotashield vaccine, was withdrawn due to the risk of intussusception in vaccine recipients (Murphy et al., 2001).

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Rotaviruses are classified into P genotypes and G serotypes, based on the genetic and antigenic diversity of the two outer capsid proteins, VP4 and VP7, respectively. These proteins independently induce type-specific neutralizing antibodies and play an important role in the development of protective immunity against rotavirus reinfection. To date, 14 G and 20 P types have been defined (Estes et al., 2001; Hoshino and Kapikian, 2000), although recent nucleotide sequence analogues of a bovine rotavirus strain indicate that there may be a 15th G serotype and a 21st P genotype (Rao et al., 2000).

The development of an effective rotavirus vaccine is challenged by the high degree of genetic and antigenic variation within rotavirus strains, similar to the case with other RNA viruses. The discovery of new rotavirus strains, reports of substantial genetic drift in predominating genotypes over time, and phylogenetic characterization of rotavirus strains provides essential information in progress toward the development of an effective vaccine against rotavirus diarrhea. Data pertaining to genetic and antigenic variation within rotavirus strains may also aid in predictions of the protective efficacy of vaccine candidates and explain the failure of some vaccine candidates.

Rotavirus vaccines are constructed to provide specific immunity against one or multiple VP7 and VP4 surface proteins. Vaccine candidates have been designed to contain either one or all of the globally most common strains, P[8], G1; P[4], G2; P[8], G3; and P[8], G4 (Kapikian et al., 2001). Rotavirus strain surveillance has intensified over the past 5 years, ensuring that the major circulating G and P antigens are being included in prototype vaccines. These efforts have been productive, as an unexpectedly high diversity of human rotavirus isolates have been reported during the past decade (Armah et al., 2001; Cunliffe et al., 2001a, 2001b; Fischer et al., 2000; Griffin et al., 2002; Ramachandran et al., 1996; Steele et al., 1999). The response to the apparent emergence of strains with the G9 serotype, first in India (Ramachandran et al., 2000) and then worldwide (Cunliffe et al., 2001a), is an example of how rotavirus surveillance data can guide vaccine development. Soon after the importance of G9 strains became clear, vaccine developers in India initiated steps to produce a vaccine with the G9 specificity (Jain et al., 2001; Ramachandran et al., 2000).

The uncommon G serotypes, most of which were originally limited to animal isolates, include G5 in pigs and G6, G8, and G10 in cattle (Palombo, 2002). An increasing number are now detected in humans, probably because of improved methods for strain characterization and due to the ability of rotavirus strains to reassort (Kapikian et al., 2001). Although interspecies transmission of rotaviruses has not been conclusively reported (Desselberger and Estes, 2000), an increasing number of reports of atypical rotavirus isolates possessing characteristics suggestive of mixed species origin and phylogenetic analyses of these strains support the notion that reassortment between progenitor viruses from

different species may occur (Nakagomi and Nakagomi, 1993). The apparent interspecies transmission of either entire viruses or of individual gene segments coupled with a high degree of genetic and antigenic variation indicate that the inclusion of a greater variety of strains into future vaccine formulations is required (Palombo, 2002).

In our previous paper reporting on rotavirus genotypes of 167 rotavirus samples from Guinea-Bissau (Fischer et al., 2000), a total of 76 samples were found to be incompletely typed. In general, the globally common genotypes were underrepresented among this population, and a substantial year-to-year diversity in locally circulating strains was observed. In 1996 and 1997, P[6], G2 was the most dominant strain, whereas P[8], G1 was the most common strain in 1998.

In order to expand on our understanding of the diversity of rotavirus strains infecting young children in one of the world's poorest countries, Guinea-Bissau, the 76 incompletely typed strains were subjected to further characterization. During the study, methodological limitations of the standard typing techniques were identified and resolved.

Results

Virus cultivation

Eleven of the 27 specimens with NT strains had sufficient material for virus cultivation (for the remaining 16 NT samples, only purified dsRNA was available for further characterization). Five of the strains were successfully cultivated as previously described (Fischer et al., 2002), and the purified dsRNA was subsequently extracted and genotyped according to methods described elsewhere.

G and P typing of rotavirus strains

The G genotype was identified in 74 (97%) of the 76 specimens. A total of 32 specimens (41%) had a single G type and of the single G types, 17% were G1, 62% were G2, and 21% were G8. A total of 45 specimens (59%) contained mixed infections, with G2G8 and G2G9 as the most frequent combinations (Fig. 1 and Table 1). The cultivated strains proved to be P[6], G2; P[4], G8; and P[6], G2G8 and two specimens yielded P[4]P[8], G2.

The P genotype could be identified in 67 samples (87%). A total of 42 (55%) specimens contained single P types, predominantly P4 or P6, whereas 25 specimens (33%) revealed mixed infections, of which 22 were P[4]P[6] types. In 9 specimens (13%), the P type could not be identified.

The 49 PT specimens were initially screened by both standard G-typing primer sets (Das et al., 1994; Gouvea et al., 1990). When the sBeg9/End9 products were used as templates in a subsequent multiplex PCR reaction with a primer mix containing the VP7-specific primers designed by Gouvea et al., 13 (27%) of the 49 PT specimens revealed a

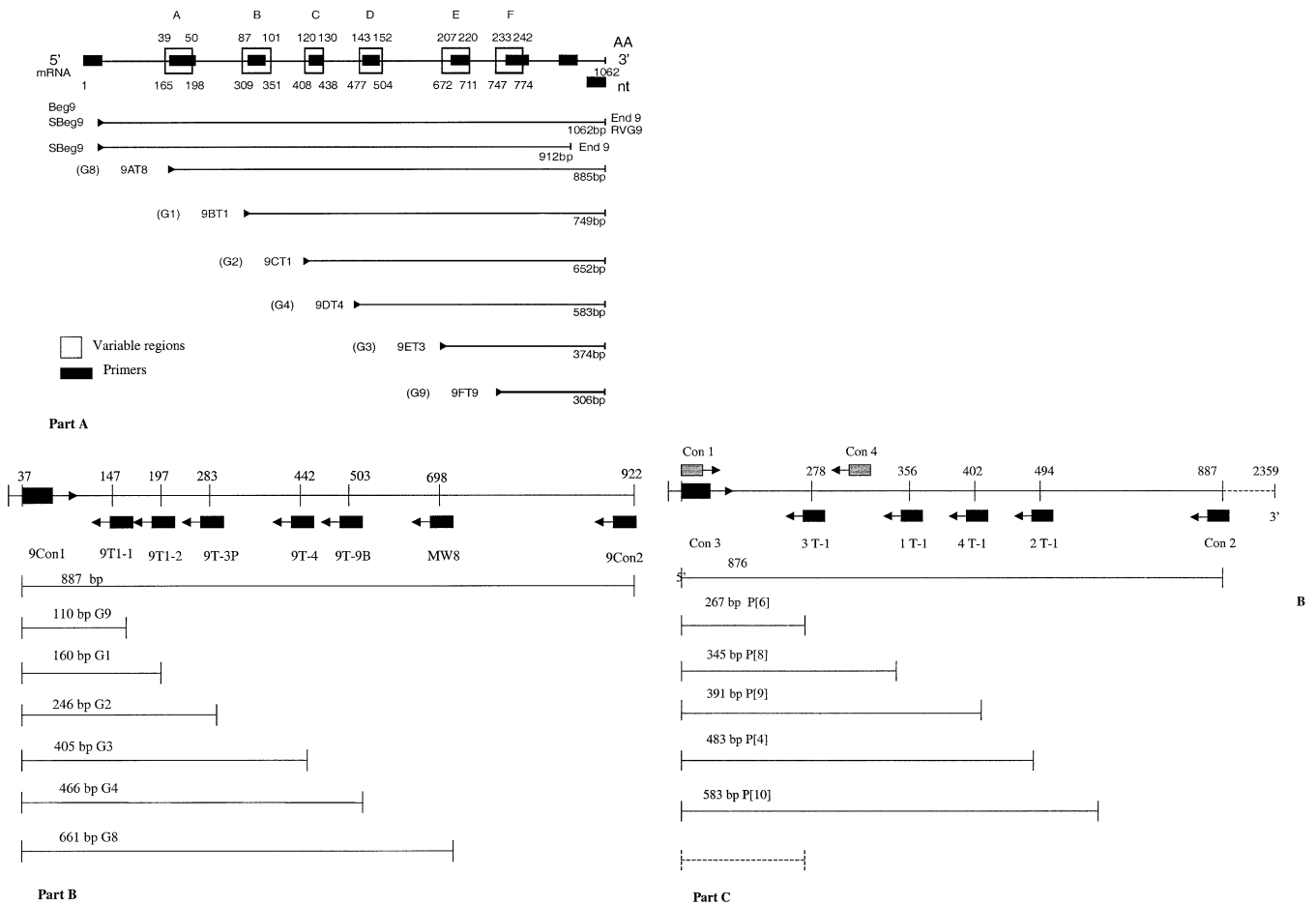


Fig. 1. (A) VP7 PCR typing of human rotavirus, with the positions and directions of amplification relative to those for the plus (mRNA) sense genomic strand for the consensus primers sBeg9 and End9 and for the gene 9 type-specific primers aAT8, aBT1, aCT2, aDT4, aET3, and aFT9 shown. The sizes of the expected products of amplification from sBeg9, End9 (first amplification), and sBeg9 plus aAT8, aBT1, aCT2, aDT4, aET3, and aFT9 (second amplification, gene 9 typing PCR) are also shown (Gouvea et al., 1990). (B) VP7 PCR typing of human rotavirus, with the positions and directions of amplification relative to those for the plus (mRNA) sense genomic strand for the consensus primers 9Con1 and 9Con2 and for the gene 9 type-specific primers, 9T1-1, 9T1-2, 9T-3P, 9T-4, 9T-9B, and MW8 shown. The sizes of the expected products of amplification from 9Con1, 9Con2 (first amplification), and 9Con1 plus 9T1-1, 9T1-2, 9T-3P, 9T-4, and 9T- (second amplification, gene 9 typing PCR) are also shown (Das et al., 1994). (C) VP4 PCR typing of human rotavirus, with the positions and directions of amplification relative to those for the plus (mRNA) sense genomic strand for the consensus primers Con3 and Con2 and for the gene 4 type-specific primers 1T-1 to 4T-1. The sizes of the expected products of amplification from Con3, Con2 (first amplification), and Con3 plus 1T-1 through 4T-1 (second amplification, gene 4 typing PCR) are also shown (Gentsch et al., 1992). In addition the size of the expected product of amplification from Con1 and Con4 is shown.

single G9, 5 (10%) a single G8, 7 (14%) mixed G3G8 infection, and only 2 specimens (4%) revealed a single G2. Five specimens remained NT and 11 specimens displayed G8 or G9 specificity. When the 9Con1/9Con2 products from the same 49 specimens were subjected to a multiplex PCR with a primer mix containing the VP7-specific primers designed by Das et al., 17 (35%) of the specimens revealed a single G2 (including the two single G2 types identified previously using the Gouvea et al. primers), 24 (49%) G2G8 mixed infections, and a few other G type combinations were identified. No G3 types were identified using the Das et al. primers.

Based on these observations, six of sBeg9/End9 products yielding a single G9 band with Gouvea et al. primers and a single G2 with Das et al. primers were rescreened using a

nested standard multiplex PCR and the Das et al. VP7-specific typing primers (Das et al., 1994). Five specimens now revealed a single G2-specific amplicon. Omission of the 9T1-1 G9 typing primer from the multiplex primer mix resulted in stronger G2 bands in five of the samples and a G2-specific product in the previous negative sample. When the samples were subsequently analyzed with only the 9T1-1 G9 typing primer and 9Con1, a G9 band was revealed in six specimens. In fact, no G9 strains were revealed among the dually infected specimens with the primers developed by Das et al. before the full-length VP7 cDNA was subjected to the single G9-specific primer in the PCR. The G2G9 mixed infections were confirmed by partial sequencing of the PCR products from two of these samples.

The remaining 43 PT samples were subjected to multi-

Table 1
The G and P genotypes of 76 rotavirus strains subjected to in-depth analysis

P, G genotypes	1996	1997	1998	1996–1998
Completely typed				
Single infections				
P[6], G1	2	0	1	3
P[8], G1	0	2	0	2
P[4], G2	3	0	0	3
P[6], G2	4	6	2	12
P[4], G8	2	1	0	3
P[6], G8	0	3	0	3
Multiple infections				
P[6]P[8], G1G8	0	0	1	1
P[4]P[6], G2	0	1	0	1
P[4]P[8], G2	2	0	0	2
P[4], G2G8	2	0	0	2
P[6], G2G8	0	9	0	9
P[4]P[6], G2G8	2	8	0	10
P[6], G2G9	2	2	0	4
P[4]P[6], G2G9	2	9	0	11
P[4], G8G9	0	1	0	1
Partially typed				
P?, G2	0	2	0	2
P?, G8	0	1	0	1
P?, G2G8	2	2	0	4
Not typeable	0	0	2	2
Total	23	47	6	76

Specimens were collected from young children in Guinea-Bissau during the period of 1996 to 1998.

plex PCR analyses using G1 to G4 serotype-specific primers 9T1-2, 9T3-P, 9T-4, and 9T-9B (Das et al., 1994) and single locus PCR analyses using the G8 (MW8) and G9 (9T1-1) primers, respectively.

Based on these analyses, 15 G2G9 mixed infections were identified. The two G2 types identified by Gouvea et al. primers were also identified as single G2 genotypes with the primers developed by Das et al.

In two samples, the G type as well as the P type remained unidentified despite cultivation and several attempts of RT priming with unconventional consensus primers (sBeg9, RVG) (Gouvea et al., 1990) as well as with various rare typing primers (G5, G12, and P14) (Browning et al., 1991; Fitzgerald et al., 1995; Gouvea et al., 1994). No first amplicon products could be obtained, and therefore these samples could not be sequenced.

Table 2

Alignments of the fragments of the VP4 gene of rotavirus strains from Guinea-Bissau and the corresponding sequences of the original 2T-1 (P[4]) and 3T-1 (P[6]) primers

Primer	Sequence	Nt position
G-BP[4] (5679 AF:)	GAT-AAT-AAT-CTT-CAA-TCT-CAA	
G-BP[4] (AF519204)	GAT-AAT-AAT-CTC-CAA-TTT-CAG	474 to 494
G-BP[4] (AF519203)	GAT-AAT-AAT-CTC-CAA-TTT-CAG	
2T-1 primer	CTA-TTG-TTA-GAG-GTT-AGA-GTC	
G-BP[6] (AF519205)	ACA-ACT-AAC-CAA-CCT-AAG-TT	246 to 266
3T-1 primer	TGT-TGA-TTA-GTT-GGA-TTC-AA	

Silent mutations are in boldface.

The G2, G8, and G9 strains were more likely to occur in mixed infections rather than in single infections ($P < 0.001$), whereas G1 strains were all single strains except for one mixed infection with P[6]P[8], G1G8 ($P < 0.001$).

There was an excellent correlation between RT-PCR for P typing using the two standard primers sets, and no interprimer suppression or cross-typing was revealed.

Primer binding site point mutations

Samples identified as mixed G3 and G8 infections by PCR using primers designed by Gouvea et al. proved to be only single infections with G8 strains when subjected to sequence analysis.

A subset of VP4-specific amplicons from isolates that could not be P typed was selected for partial sequencing of the VP4 gene. The nucleotide sequence alignment showed one-point mutations at the primer binding site of the P[6]-specific primer (3T-1) (at nt 254) and at the primer binding site of the P[4]-specific primer (2T-1) (at nt 488), respectively (Table 2).

Phylogenetic relationships

We obtained complete VP7 sequences (1062 bp) for two Guinean G8 strains and partial G8 sequences for another four strains; only the complete sequences were included in the final phylogenetic analyses. Our Guinean G8 strains were 98% identical to the Nigerian HMG89 (X98918) strain and 97% identical to the Malawian MW333 (AJ278257) strain (Fig. 2). Our G9 strains were 98% identical to the Nigerian Bulumkutu strain (AF359358) and 97% identical to the Malawian MW69 strain (AJ250545).

Discussion

This study reports upon the in-depth analysis of 76 IT Guinean rotavirus-positive specimens. The study met with methodological challenges such as cross-priming and interprimer suppression when employing a standard multiplex RT-PCR procedure with the two most commonly used primer pair sets (Das et al., 1994; Gouvea et al., 1990). The

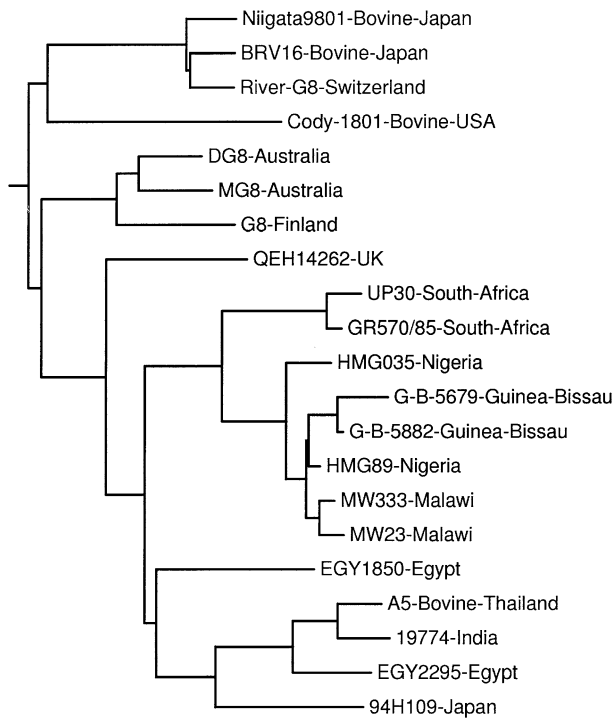


Fig. 2. Phylogenetic tree based on the VP7 proteins of G8 rotavirus serotypes. Sequences were multiple aligned using clustalw, and the tree was constructed using neighbor joining ignoring all positions with gaps. The tree was rooted on an outgroup consisting of bovine sequences from Japan and the United States and of a sequence isolated from a Swiss river. The following G8 sequences were taken from the GenBank database (Accession numbers): Niigata9801 (AB044293), BRV16 (BAB83660), River G8 (AAC27739), Cody 1801 (AAA87710), DG8 (AF034852), MG8 (AAF23774), G8 Finland (Q9UBA6), QEHI4262 (AF143689), UP30 (AF143690), GR570/85 (AAD33919), HMG035 (AAK66979), HMG89 (AF CAA67420), MW333 (CAB 92924), MW23 (CAB92921), EGY1850 (AF104102), A5 (D13395), 19774 (AAG31659), EGY2295 (AF104104), and 94H109 (BAB40362).

observed complexities were solved by modifications including the use of G8 and G9 typing primers (along with the consensus primers) in separate PCR reactions and the application of a second set of typing primers when unexpected PCR products were obtained. Separate use of the 9T1-1 G9 typing primer increased the number of G2 and G9 strains identified, as inclusion of this primers in the standard G1–G4 + G9 multiplex primer mixture (Das et al., 1994; Gouvea et al., 1990) seemed to interfere with amplification of the G2-specific sequences. Cultivation of NT specimens, the use of the unconventional primers sBeg9, RVG, Con1, and Con4 for first round PCR amplification of the VP4 and VP7 genes, and the use of rare primers (G5, G8, G12, P14) during second round PCR resulted in a reduction in the percentage of NT strains from 16 to 3%.

The high prevalence of G8 strains among the NT specimens suggests that a dual infection between G8 and G2 strains somehow inhibits the amplification of the G2 strain. In fact, some samples were only possible to type after several attempts and some only when preceded by virus

strain cultivation. The emergence of G8 and G9 strains in Guinea-Bissau is not surprising, considering the recent reports of this globally rather uncommon strain from other rotavirus surveillance sites in Africa (Armah et al., 2001; Cunliffe et al., 2001b, 2002; Ramachandran et al., 1996; Steele et al., 1999).

Cross-priming of G3 and G8 strains was detected coincidentally, as the G3 strains only appeared during application of one of the two standard primer sets. Sequence analysis of the Guinean G8 strains revealed a single nucleotide mutation at the 3' end of the aAT8 (G8) primer binding site that explains the failure of this primer to detect the Guinean G8 strains. Similar results were found in Nigeria, where G8 mispriming was most probably due to mutations at the 3' end of the primer binding site (Adah et al., 1997). It was therefore not surprising that the Guinean G8 strains demonstrated high identity with the Nigerian HMG89 strain. The high degree of identity between the Malawian G8 strains and the Guinea-Bissau G8 strains probably accounts for the success of primer MW-8 (Cunliffe et al., 2000), which was originally developed for identification of G8 strains in Malawi. The observations highlight the need for a follow up of unexpected findings, either using another set of typing primers or, ultimately, by nucleotide sequence analysis.

The high frequency of mixed infections (59%) detected during the in-depth analyses of the incompletely typed samples indicates that the standard multiplex RT-PCR typing method may not work optimally in analysis of multiple infections. There may be several explanations for this observation: some are related to the primers, such as interprimer suppression (dimerization) and/or competition and/or that the applied annealing temperature during the standard multiplex PCR is closer to that which is optimal for one of the primers than for the other. Another explanation is related to the viral dsRNA, as different concentrations of virus could play a role in the case of mixed infections, considering that the most abundant dsRNA (e.g., G8 or G9) out-competes a low-concentration dsRNA (e.g., G2).

Another feature of the mixed infections was that approximately 50% of these samples revealed a “double-double” infection, i.e., the combination of two P and two G types identified in one specimen, the most frequent being the [P4] [P6], G2G9 combination, representing 20% of all mixed infections. To our knowledge, the genotypic combinations of a mixed infection, [P4] [P6], G2G8 and [P4] [P6], G2G9, have not been described previously. In general, mixed infections have so far comprised a limited proportion of rotavirus positive specimens and have been detected at a frequency ranging between 1 and 12% (Asmah et al., 2001; Cunliffe et al., 1998; Griffin et al., 2002; Rodriguez et al., 2000). Unless the methodological limitations observed of the standard typing techniques is a local phenomenon exclusively occurring in our Guinean rotavirus strains, our findings cast some doubt over the true frequency of multiple

infections reported in other studies, where standard PCR procedures with a multiple primer pool are used.

The frequent detections of the “double–double” infections supports the hypothesis previously generated by others that rotavirus exists as heterogeneous populations of reassortants (Gouvea and Brantly, 1995); thus the uncommon [P4] [P6], G2G8 and [P4] [P6], G2G9 mixed infections can be explained by natural reassortment of common P[6]G8 and P[4]G2 strains and P[6]G9 and P[4]G2 strains, respectively.

Point mutations at the primer binding sites of the P[4]- and P[6]-specific primers (IT-2 and 1T-3, respectively) decreased the affinity of primer binding and may explain the failure to identify these P[4] and P[6] strains during the initial RT–PCR. Specifically these two strains displayed two point mutations in the P[4] primer binding site and one point mutation in the P[6] primer binding site (Table 2). Analysis of the VP4 reading frame showed that all three mutations were placed in third positions of the codons and that they were silent. It is not surprising that the mutations detected in the primer binding sites were all silent as these sites have been chosen in the most highly conserved regions of the rotavirus genome; there is accordingly strong selection to maintain the encoded amino acids at these positions. Our observations underscore the need for regular evaluation of the applied typing primers. Moreover, the rapid evolution of rotavirus combined with the fact that there is usually no selection against silent mutations suggests that it might be an advantage to take the degeneracy of the genetic code into account when designing typing primers. A similar phenomenon was described in the United Kingdom, where a degenerate version (1T-1D) of the P[8]-specific primer (1T-1) allowed strains previously NT due to point mutations at the primer binding site to be P typed by RT–PCR (Iturriza-Gomara et al., 2000).

The majority of the strains were characterized through analysis based upon modifications of the standard procedures and/or by partial sequencing of either the amplicons obtained from using VP7 or VP4 consensus primers in the first round RT–PCR or the type-specific amplicons obtained from the second round of type-specific PCR.

In conclusion, through in-depth studies of IT rotavirus strains, difficulties in the conventional RT–PCR typing procedures were identified and resolved by (1) modifications of the standard procedures, (2) the use of uncommon consensus and type-specific primers, as well as (3) the use of more comprehensive methods, such as virus cultivation, nucleotide sequence analyses, and phylogenetic analyses. The high frequency of mixed infections identified among the IT samples is likely to facilitate frequent reassortment of the virus genome. Based on the high prevalence of the otherwise rather uncommon G8 and G9 strains, we suggest that consideration should be given to incorporating such strains into existing vaccine candidates and in the next generation rotavirus vaccine targeted for the African continent, especially

if the vaccine candidates currently undergoing testing fails to provide satisfactory protection against rotavirus diarrhea.

Materials and methods

Rotavirus specimens

As previously described (Fischer et al., 2000), specimen collection was undertaken during epidemiological studies in four suburban districts of the capital Bissau, Guinea-Bissau, from January 1996 to April 1998. During 1996–1998, 203 rotavirus-positive stool samples were tested positive with a commercial ELISA kit (DAKO, Copenhagen, Denmark). The specimens were collected from young children residing in a suburban area in Guinea-Bissau, West Africa, and 167 of these samples that were not repeated isolates from continuous infections contained sufficient material for further analyses. Eighty-seven (52%) samples were characterized, according to G and P specificities, while 80 specimens could not be completely characterized. Seventy-six of these incompletely typed (IT) specimens contained sufficient material for further characterization and were split into two groups. The first group contained 49 (32%) partially typed (PT) samples, i.e., only one of the two genotypic specificities had been obtained, and the second group consisted of 27 (16%) nontypeable (NT) samples, i.e., neither a G nor a P genotype was identified. Unlike the first Guinean study, which utilized a one-step multiplex RT–PCR method developed by Das et al. (1994), IT specimens in this study were subjected to the two step RT–PCR procedure described by Gouvea et al. (1990) for VP7 genotyping and by Gentsch et al. (1992) for VP4 genotyping.

Cultivation of strains

Eleven of the 27 NT samples had sufficient stool material for cell cultivation. Specimens suspended in PBS were activated with trypsin and passaged three times in roller tubes of monkey kidney cells (MA104) by established methods (Mason et al., 1980).

RNA extraction

A commercial RNaid kit (Bio 101, Inc., La Jolla, CA) was used for extraction of dsRNA from stool, similar to the procedure described previously (Fischer et al., 2000). Specimens subjected to virus cultivation were extracted from lysates of rotavirus-infected MA104 cells with phenol–chloroform–1% sodium and ETOH precipitation, utilizing the method described by Gouvea et al. (1990).

VP7-specific primers

Generic primers sBeg9/End9 (Gouvea et al., 1990) were used to produce full-length copies (1062 bp) of gene 9 when

amplification using the standard 9Con1/9Con2 consensus primers failed (Figs. 1A and B). All the 76 specimens were screened with the serotype-specific primers 9T1-1, 9T1-2, 9T3-P, 9T-4, and 9T-9B (Das et al., 1994) in nested multiplex PCR reactions and the MW8 serotype-specific primer in single locus PCR reactions (Cunliffe et al., 2000). The 49 PT specimens were additionally screened with the serotype-specific aBT1, aCT2, aET3, aDT4, aAT8, and AFT9 (Gouvea et al., 1990) in nested multiplex PCR reactions (Figs. 1A and B). The 9Con1 primer was used in all nested PCRs in combination with the type-specific primers.

VP4-specific primers

Amplification of shorter highly conserved regions on gene 4, using Con1 (5'-TTGCCACCAATTCAAATAC-3') and Con4 (5'-TATTACATTGCATTTCTTTCCA-3') primers was performed when amplification with Con2/Con3 failed. The short Con1/Con4 products were subjected to nucleotide sequencing. The 1T-1 to 5T-1 type-specific primers (Gentsch et al., 1992) were used against the Con3 primer in all second round heminested PCRs (Fig. 1C).

RT-PCR reactions and G- and P-typing nested PCRs

A two-step RT-PCR procedure was applied, slightly modified from that described previously (Fischer et al., 2000). First, the duration of the RT reaction was prolonged from 30 to 60 min and second, the type-specific primers G8 and G9 were not added to the standard multiplex type-specific primer mix, but used with a consensus primer one at a time in separate PCR reactions.

Agarose gel analysis

The first step amplicons (sBeg9/End9, Con3/Con2, or Con1/Con4 products) were run on a 1% agarose gel, and the second step amplicons (all type-specific PCR products) on a 3% agarose gel (Sigma Chemical Co., St. Louis MO) and bands were visualized by ethidium bromide staining.

Cloning and sequencing

Selected VP7 and VP4 amplicons from extracted cell lysates were sequenced directly to verify the authenticity of the results obtained during G and P typing. VP7 gene RT-PCR products of serotypes G2, G8, and G9 were synthesized with the 9Con1/G2, 9Con1/G8, and 9Con1/G9 primers, respectively. The VP4 gene RT-PCR products were synthesized with Con3/Con2 or Con1/Con4 primer pairs. The PCR products were excised from a low-melting 1.2% SeaKem GTG agarose gel (FMC Bioproducts, Rockland ME), purified using the QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA), and sequenced with the appropriate primers using the Prism Ready BIG DYE Terminator Cycle Sequencing kit (PE Applied Biosystems, Foster City,

CA) using an automated sequencer (Applied Biosystems Model 377) under the thermal cycling conditions recommended by the manufacturer.

Amplicons from the PT specimens were cloned into the TOPO 4 cloning vector (Invitrogen, Groningen, The Netherlands) before sequencing. Cloning of VP4 and VP7 gene RT-PCR products was performed by inserting either the full-length Con3/Con2 (876 bp) or sBeg9/End9 (1062 bp) products or serotype-specific PCR products into the TOPO 4 cloning vector (Invitrogen). The cloned inserts were cycle-sequenced in both directions using IR-labeled M13 primers and the Thermo Sequenase DYEnamic Direct cycle sequencing kit (Amersham Biosciences, Piscataway, NJ). Sequence reactions were separated by electrophoresis on 66-cm glass plates using a LI-COR sequencer (LI-COR, Lincoln, NE). The sequence data obtained were then compared against the GenBank database using the BLAST suite of programs. The Clustalw program (Thompson et al., 1994) was used to construct both multiple alignment and neighbor joining trees, ignoring all alignment positions with gaps.

Nucleotide sequence Accession numbers

The Guinean VP7 and VP4 gene sequences described in this study have been deposited in the GenBank sequence database. The partial VP7 gene sequences of Guinean G2 strains have been assigned Nos. AF516716, AF516718, and AF519207 and the complete VP7 gene of a Guinean G2 has been assigned No. AF520961. The partial VP7 gene sequences of Guinean G8 and G9 strains have been assigned Nos. AF520962 and AF520963 for G8 and AF516715 and AF519206 for G9, respectively. The partial VP4 gene sequences of Guinean P[4] strains have been assigned Nos. AF516717, AF519203, and AF519204. The partial VP4 gene sequence of a Guinean P[6] strain has been assigned No. AF519205.

Statistics

The χ^2 test was used to test the probability of achieving single versus mixed infections. The statistical analyses were performed using the SAS System, version 8.2, for windows (SAS Institute Inc., Cary, NC).

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