SEQUENCE ANALYSIS OF VP7 GENE OF TWO NIGERIAN ROTAVIRUS STRAINS

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Received July 9, 1996; revised August 22, 1996

Summary. – The complete nucleotide sequences of gene 9 (VP7) of rotavirus strains MGH66 and RHIB55 isolated in northern and southern Nigeria, respectively, were determined. The sequence of either strain was 1062 nucleotides long with two potential glycosylation sites and two in-phase initiation codons encoding a protein of 326 amino acids provided the first ATG codon was utilised. Comparison of the deduced amino acid sequences of VP7 of the strains with that of published sequences of serotype G1 strains and a representative strain of each of serotypes 2-6 and 8-14 revealed ≥91.41% and ≥81.60% homology, respectively. The stool sample obtained from a diarrhoeic child in Maiduguri containing strain MGH66 was classified by polymerase chain reaction (PCR) technique as possessing a dual infection specificity of VP7 serotypes G1 and G3. The nucleotide sequencing, however, revealed the dual infection specificity of VP7 serotypes G1 and G8. The implications of nucleotide sequence analysis for serotyping of rotavirus strains originating from different geographical regions and for vaccine development are discussed.

Key words: rotavirus; VP7; Nigeria; nucleotide sequence analysis

Introduction

Ever since rotavirus has been identified as an important cause of diarrhoea in infants and children world-wide (Kapikian and Chanock, 1996), the need to develop a vaccine against it has been a major focus of the rotavirus research. Rotaviruses of serotype 1 are the single most important cause of diarrhoeal disease throughout the world (Bishop *et al.*, 1991), though recent reports from some developing countries have indicated that also other serotypes could account for a significant portion of these infections (Ramachandran *et al.*, 1996; Timenetsky *et al.*, 1994; Steele *et al.*, 1995; Aijaz *et al.*, 1996). Moreover, the evaluation of the efficacy trial of some rotavirus vaccine candidates has shown a varying degree of success across age and geographical regions (Christy *et al.*, 1988; Clark *et al.*, 1988; DeMol *et al.*, 1986; Flores *et al.*, 1987; Hanlon *et al.*, 1987; Kapikian *et al.*, 1985,1986, 1988; Rennels *et al.*, 1986; Vesikari *et al.*, 1984, 1985).

Only recently we have reported our findings on the molecular characterisation of the gene 9 of rotavirus strains from Nigeria and found a significant level of unusual characteristics (Adah *et al.*, 1996). Further analysis of the samples revealed that rotavirus strains bearing VP7 serotype G8 could not be typed by PCR due to nucleotide substitutions at the primer binding site.

Since amino acid differences in both VP4 and VP7 between different isolates from different geographical locations are associated with genetic diversity (Kirkwood *et al.*, 1996) and sequence data on Nigerian rotavirus strains are not available, we sequenced the complete gene 9 of two serotype G1 rotavirus strains from Nigeria. In this report,

Abbreviations: aa = amino acid; DMSO = dimethyl sulphoxide; MLV = murine leukemia virus; nt = nucleotide; ORF = open reading frame; PCR = polymerase chain reaction; RT = reverse transcription

we also present confirmation of dual infection specificity by nucleotide sequence analysis.

Materials and Methods

Viruses. The rotavirus strains MGH66 and RHIB55 were isolated from children in northern and southern Nigeria, respectively, and previously characterised for their G and P types (Adah *et al.*, 1996). The stool sample, which strain MGH66 was identified from, was classified by PCR as possessing dual infection specificity of both G1 and G3 types, and single specificity of P6 type. Strain RHIB55 was of G1P8 type. MGH66 had short electrophoretype, while RHIB55 had a long one.

Reverse transcription/Polymerase chain reaction (RT/PCR). A slight modification of the method of Gouvea *et al.* (1990) was used for the RT/PCR protocol. The primers were Beg9 [5'GGCTTTAAAAGAGAGAATTTCCGTCTGG3'] and End9[5'GGTCACATCATACAATTCTAATCTAAG3']. Double-stranded RNA was extracted from stool specimens according to the method of Herring *et al.* (1982), purified with RNAid PLUS kit (Dianova, Germany) according to the manufacturer's instructions and used as template for RT/PCR. RNA (50 ng – 1 μg) was mixed with 7% dimethyl sulphoxide (DMSO) and 100 pmoles of each primer in a total volume of 50 μl. It was overlaid with 100 μl of mineral oil and the mixture was heated at 95°C for 3 mins and then cooled to 37°C in a programmable thermal controller PTC-100 (Biozym, Germany.) One volume of 2-fold RT/PCR buffer containing dCTP, dTTP, dATP and dGTP (5 mmol/l each), 2 U of Taq polymerase (Gibco/BRL) and 100 U of MLV reverse transcriptase was added and the mixture was incubated at 37°C for 40 mins. The 2-fold RT/PCR buffer consisted of 100 mmol/l Tris-HCl pH 8.0, 3 mmol/l $MgCl_2$ and 40 mmol/l KCl. The PCR programme consisted of 30 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 2 mins, and of a final incubation at 72°C for 10 mins.

Second amplification and serotyping. In the second amplification reaction and typing, 5μl of the RT/PCR amplification product was used as template and the reverse transcriptase was omitted. The template was mixed with 100 pmoles each of the serotype-specific primers and the common primer End9, 3 mmol/l $MgCl_2$, dATP, dCTP, dTTP and dGTP (5 mmol/l each), and 1 U of Taq polymerase in PCR buffer in a final volume of 100 μl. This was overlaid with 100 μl of mineral oil and the same PCR protocol was repeated. The serotype-specific primers used in the reaction included aAT8 (5'GTCACACCATTTGTAAATTCG3', serotype G8 primer), aBT1 (5'CAAGTACTCAAATCAATGATGG3', serotype G1 primer), aET3 (5'CGTTTGAAGAAGTTGCAACAG3', serotype G3 primer), and PR18 (5'GTCACACCATTCGTAAACTCA3',serotype G8 primer specific for Nigerian strains).

Nucleotide sequencing. Two clones each from independent PCR products were sequenced for each of the strains to obtain the final version of the nucleotide sequence. The sequencing was carried out in both directions to obtain the full length gene 9 sequence of both the positive and negative strands of cDNA. The primers used in this study included PRI2 (5'GGTGAATGGAGAGACTCATTGTCGC3') for the positive strand, and PRI3 (3'CAGCAACCATCTCAAATGAGTCTA5') for the negative strand. The cloning of rotavirus cDNA, transformation of component cells, nucleotide sequencing and confirmatory analysis of recombinant clones were carried out according to the methods of Marchuk *et al.* (1991), Bolivar *et al.* (1977) and Sanger *et al.* (1977), using commercial T-cloning kit (MBI Fermentas, Germany) and ABI Prism™ Dye Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase, FS (P/N 402078, Revision A, August 1995, Perkin Elmer, USA). Gel electrophoresis and sequencing analysis was carried out on automatic DNA sequencer, model 373 DNA (Applied Biosystems, USA). In addition, following the serotyping PCR of the sample containing strain MGH66, the G1 and G3 reamplification products were isolated, cloned and sequenced as described above. The sequence data on the G1 and G3 reamplification products were compared with the complete gene 9 nucleotide sequence of strains MGH66 and HMG89, and that of reference laboratory strains Wa, HCR3 and 69M. The nucleotide and deduced amino acid sequences of strains MGH66 and RHIB55 were compared with published sequences of serotype G1 strains Wa, D, Mo, M37, RV-4 and of Nigerian serotype G8 strain HMG89 using computer programme HIBIO DNASISTM (Hitachi America, USA), Clustal W 1.5 (Thompson *et al.*, 1994) and TreeView 16 (Page, 1996).

Results

Nucleotide sequence analysis

The nucleotide sequences of the two strains are presented in Fig. 1. It should be stressed that two clones each from two independent PCR products were sequenced for each of the strains to obtain the final version of the nucleotide sequence, and that the same was true for the G1 and G3 reamplification products of the sample containing strain MGH66.

The structural organisation of the gene in both strains was similar to that of other known serotype G1 strains. Like in other serotype G1 strains, VP7 gene of both strains was 1062 nucleotides in length with two in-phase initiation codons located at nt 49-51 and 136-138. The longest open reading frame (ORF) could encode a protein of 326 amino acids. The presence and location of cysteins (8 cysteins were conserved in all strains) and prolines (9 prolines were also conserved in

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RHIB55      1     GGCTTTAAAAGAGAGAATTTCCGTCTGACTAACGGTTAGCTCCTTTTGATGTATGGTATT 60
MGH66       1     ----------------------------G-------------------------------- 60

RHIB55      61    GAATATACCACAATTCTAATCTTTCTGATATCAATCATTCTACTCAACTATATGTTAAAA 120
MGH66       61    ------------------------------------------------------------ 120

RHIB55      121   TCAGTGACCCGAATGATGGACTACATTATATATAGATTTTTGTTAATTTCTGTAGCATTA 180
MGH66       121   ----------------------------------------------G----------- 180

RHIB55      181   TTTGCCTTGACAAAAGCTCAGAATTATGGACTTAACATACCAATAACAGGATCAATGGAC 240
MGH66       181   ------C----------------------T---------------------------- 240

RHIB55      241   ACTGTATACTCCAACTCTACTCAAGAAGGAATATTTCTAACATCTACATTATGTTTGTAC 300
MGH66       241   ------------------------------------------------------A--- 300

RHIB55      301   TATCCAACTGAAGCAAGTACTCAAATCAATGATGGTGAATGGAGAGACTCATTGTCGCAA 360
MGH66       301   ----------------------------C----------------------------- 360

RHIB55      361   ATGTTTCTTACGAAAGGTTGGCCAACAGGATCAGTCTATTTTAAAGAGTACTTAAATATT 420
MGH66       361   ------------------------------------------------------C--- 420

RHIB55      421   GTTGATTTTTCTGTTGATCCACAATTGTATTGTGATTATAACTTAGTACTAATGAAATAT 480
MGH66       421   ----------------C--G----------C-------------------------- 480

RHIB55      481   GATCAAAATCTTGAACTAGATATGTCAGAATTGGCTGATTTAATATTGAATGAATGGTTA 540
MGH66       481   --------------------------------A--------------------------- 540

RHIB55      541   TGTAATCCAATGGATATAACATTATATTATTATCAACAATCGGGAGAATCAAATAAGTGG 600
MGH66       541   ----------------------------------G----------------------- 600

RHIB55      601   ATATCAATGGGATCATCATGTACTGTGAAAGTGTGTCCACTAAATACACAAACGCTAGGA 660
MGH66       601   ------------------------------------------------------------ 660

RHIB55      661   ATAGGTTGTCAAACAGCGAATGTAGACTCATTTGAAATGGTTGCTGAGAATGAGAAATTA 720
MGH66       661   --------------A-----------------------------------A------ 720

RHIB55      721   GCTATAGTAGATGTAGTTGATGGGATAAATCATAAGATAAATTTGACAACTACGACATGT 780
MGH66       721   ----------------------------------A------------------------ 780

RHIB55      781   ACTATTCGAAATTGTAAGAAATTAGGTCCAAGAGAGAATGTAGCTGTAATACAAGTTGGT 840
MGH66       781   --------------C--------------------------------------------- 840

RHIB55      841   GGTTCTAATATATTAGACATAACAGCAGATCCAACGACTAATCCACAAATTGAGAGAATG 900
MGH66       841   ------------------------------------------------------------ 900

RHIB55      901   ATGAGAGTGAATTGGAAAAGATGGTGGCAAGTATTTTATACTATAGTAGATTATATTAAT 960
MGH66       901   ------------------------------------------------------------ 960

RHIB55      961   CAGATTGTACAGGTAATGTCCAAAAGATCAAGATCATTAAATTCTGCTGCTTTTTATTAT 1020
MGH66       961   ------------------------------------------------------------ 1020

RHIB55      1021  AGAGTGTAGATATATCTTAGATTAGAATTGTATGATGTGACC 1062
MGH66       1021  ---A-A---------------------------------- 1062
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Fig. 1
Comparison of complete nucleotide sequences of Nigerian rotavirus strains RHIB55 and MGH66
Nucleotide sequences are numbered from the 5'- to the 3'-end.

all strains) were similar to that of other published rotavirus strains (Green *et al.*, 1987, 1988; Kobayashi *et al.*, 1991; Blackhall *et al.*, 1992). Two potential glycosylation sites (at aa 69-71 and 238-240) were also conserved.

Amino acid sequence analysis

The amino acid sequence of VP7 glycoprotein of the two strains and that of other serotype G1 strains (Wa, Ku, D, Mo,

M37, RV-4 and T449) was deduced from ORF of 978 nucleotides, located at nt 49-1026. Overall, a homology of at least 91.41% or higher was observed between the compared strains (Table 1). Nigerian strains MGH66 and RHIB55 showed the highest homology of 98.47% with each other and 95.71% with human serotype G1 strain RV-4, but the lowest homology with the animal serotype strain T499. In contrast, when compared to strains representing each of the serotypes 2-6 and 8-14, a maximum homology of 81.60% was observed. Comparison of the deduced VP7 amino acid sequences of regions A, B, and C (Green *et al.*, 1989; Dyall-Smith *et al.*, 1986) of both strains with published sequences of serotype G1 strains Wa, Ku, D, Mo, M37, RV-4 and T449 revealed high level of conservation confirming the identity of the strains (Fig. 2). On the other hand, when the sequences of the two strains were compared with those of other serotypes, multiple substitutions of more than three amino acids in each of the regions were observed (data not shown). Fig. 3 shows the position of Nigerian strains MGH66 and RHIB55 in relation to other serotype strains in the phylogenetic tree.

Table 1. Comparison of VP7 amino acid sequences of Nigerian rotavirus strains MGH66 and RHIB55 with those of other strains

Strain[a] (serotype)	Amino acid homology (%)	
	MHG66	RHIB55
Wa (G1)	94.17	94.17
Ku (G1)	94.48	94.48
D (G1)	93.56	93.56
Mo (G1)	93.56	93.56
M37 (G1)	94.17	94.17
RV-4 (G1)	95.71	95.71
T429 (G1)	91.41	91.41
Sa (G2)	74.23	73.62
HCR3 (G3)	81.60	81.60
ST3 (G4)	76.38	76.07
OSU (G5)	78.83	78.83
NCDV (G6)	80.06	80.06
HMG89 (G8) (Nigerian strain)	75.77	75.77
69M (G8)	75.46	74.85
WI61 (G9)	79.45	79.45
Mc35 (G10)	77.61	77.61
YM (G11)	78.83	78.22
L26 (G12)	75.77	75.15
L338 (G13)	73.93	73.62
CH3 (G14)	78.22	78.22

[a]The corresponding GeneBank accession numbers or literature sources used in this table were as follows: Wa: K02033; T449, Wa, Ku, D, Mo, M37 and RV-4: Blackhall *et al.* (1992); S2: M11164; HCR3: L21666; ST3: X13603; OSU: X06722; NCDV: M12394; 69M and WI61: Green *et al.* (1989); Mc35: D14033; YM: M23194; L26: M58290; L338: D13549; CH3: D25229.

Confirmation of dual infection specificities by sequence analysis

The nucleotide sequence of the cloned G1 reamplification product of the sample containing strain MGH66 was compared with the complete gene 9 sequence of strain MGH66. It revealed a homology of 99.9%. In contrast, comparison of the nucleotide sequence of the cloned G3 reamplification product of the sample with the complete gene 9 sequence of strain MGH66 showed a homology of 77.5% only. On the other hand, a homology of 81.60% and 98.1% was obtained when the nucleotide sequence of the cloned G3 reamplification product was compared with the complete gene 9 sequences of serotype G3 strain HCR3 and Nigerian serotype G8 strain HMG89, respectively. These levels of homology confirm that the stool sample contained probably the strain MGH66 of serotype G1 and another strain of serotype G8.

Discussion

In this report, the sequence data of VP7 serotype G1 rotavirus strains isolated in Nigeria are presented for the first time. The data correlate with those of other workers (Green *et al.*, 1987, 1988; Taniguchi *et al.*, 1988). However, the Nigerian rotavirus strains MGH66 and RHIB55, though obtained from patients in different geographical regions of the country, widely separated apart (northern and southern Nigeria), are closely related (amino acid homology of 98.47%) to each other and to the VP7 serotype RV-4 isolate from Australia. In view of this sequence data information, it is likely that any vaccine against rotavirus that can protect infants and children against infection with G1 serotype will be effective in protecting Nigerian children against similar infection.

In the region A (according to the criteria of Dyall-Smith *et al.*, 1986) of both strains, just one amino acid substitution at residue 99 (Lys->Arg) was observed. This residue is conserved among all serotype G1 strains but the number of amino acid substitutions is not higher than two for the whole region A (Dyall-Smith *et al.*, 1986). Also, since a similar substitution at aa 97 (Asp->Glu) among M37, RV-4, T449 and the other G1 serotype strains was observed despite they all still belong to the same serotype G1, it is not likely that these changes may have any effect on the serotype specificity. Moreover, since point mutations caused by Taq polymerase cannot be excluded and assuming the error rate of Taq polymerase in PCR of one per 9000 nucleotides polymerized (Tindall and Kunkel, 1988), the overall rate of misincorporation after 30 cycles of amplification can reach 0.25% (Saiki *et al.*, 1988). However, it is noteworthy that

	REGION A aa87 → aa101	REGION B aa142→aa152	REGION C aa211 → aa223
hu/MGH66	TEASTQINDGEWRDS	MKYDQNLELDM	NVDSFEMVAENEK
hu/RHIB55	---------------	----------	----------
hu/WA	----------D-K--	-----S-----	-------I-----
hu/KU	----------D-K--	-----S-----	-------I-----
hu/D	----------D-K--	-----S-----	-------I-----
hu/MO	----------D-K--	-----S-----	-------I-----
hu/M37	-------S----K--	----------	----------
hu/RV-4	----------K--	----------	----------
bo/T449	V---N-------K-T	----------	QCG---I------

Fig. 2
Comparison of VP7 amino acid sequences of Nigerian rotavirus strains RHIB55 and MGH66 with those of other VP7 serotype G1 strains in A, B and C regions

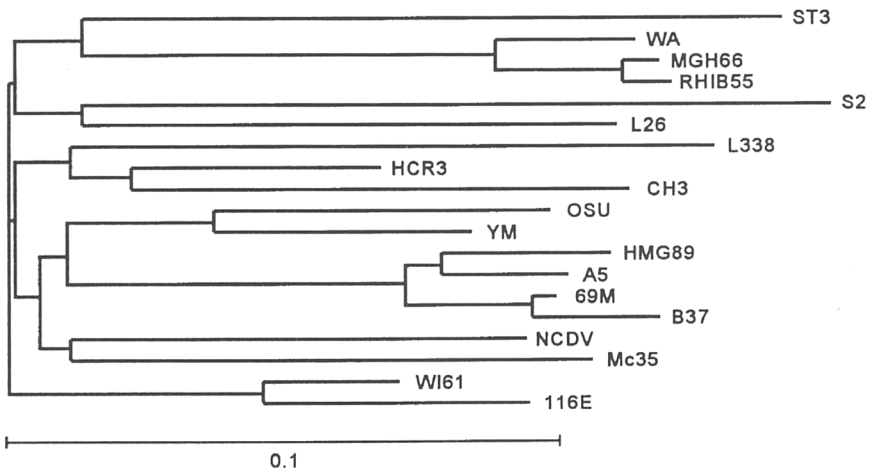

Fig. 3
Phylogenetic tree of rotavirus strains

our strains were found more closely related to strains RV-4 and M37 (strains known to be more diverse than the other serotype G1 strains) than to other strains. These data thus confirm the serotype specificity of the two strains analysed in this study.

The stool sample containing strain MGH66 was initially erroneously identified by PCR to have dual infection specificity of VP7 serotypes G1 and G3. Other workers (Steele *et al.*, 1995; Husain *et al.*, 1996) have failed to identify VP7 serotype G8 in their samples or observed intertypic sequence homologies within variable region F (between serotypes G8 and G5) and E (between serotypes G8 and G3) (Gouvea *et al.*, 1994; Hum *et al.*, 1989); this could account for false positive typing of the serotypes. After it was disclosed that Nigerian rotavirus strains belonging to Vp7 serotype G8 could not be recognised by primer aAT8, used in the serotyping assay according to Gouvea *et al.* (1990), or were erroneously classified as VP7 G3 serotype, all the suspect strains (samples) were retested for VP7 serotype G8. The sample containing strain MGH66 was one of those retested with the new primer because it had a mixed infection specificity of VP7 serotypes G1 and G3. However, it failed to be identified as a serotype G8. The comparison of the nucleotide sequence of the cloned G3 reamplification product of the sample with the complete gene 9 sequence of strain MGH66 revealed a homology of 77.5% only. In fact, it confirmed their non-identity. Since the fragment size corresponded to a VP7 serotype G3, its sequence was also compared with the complete gene 9 sequence of VP7 serotype G3 strain HCR3, and a homology of only 81.60% was observed. In contrast, the comparison of the sequence of this fragment with the complete gene 9 sequence of VP7 serotype G8 strain HMG89 from Nigeria revealed a homology of 98.1%. This clearly confirmed the dual infection specificity of the sample of VP7 serotypes G1 and G8. Our failure to identify this serotype G8, using the new primer designed on the basis of the sequence data of Nigerian strain HMG89, could be attributable perhaps to a very

low concentration of viral RNA of the serotype G8 in the samples compared to the G1 strain. We have previously tested several samples using serotype G8 radiolabelled probes and observed very faint signals after a very long exposure of the film (data not shown). Thus we classified such samples as negative for VP7 serotype G8 in accord with unequivocal results of the PCR serotyping assay. It is now clear from the sequence data analysis that the sample containing strain MGH66 has dual infection specificity of VP7 serotypes G1 and G8.

This study presents for the first time the sequence data on rotavirus VP7 serotype G1 isolated in Nigeria. In addition, it demonstrates how the sequence data analysis could be used to confirm a mixed infection with two rotaviruses in a single patient. It also confirms the presence of VP7 serotype G8 in the country. However, further studies should be undertaken to determine the extent of prevalence of serotype G8 and possibly G9 in the country. Nonetheless, any vaccine application in the country should take in consideration VP7 serotype G8, because such vaccines are developed and applied on the basis of epidemiological and molecular characteristics of the outer capsid protein of the virus known to be involved in the induction of immunity.

Acknowledgements. This study was supported in part by an University of Maiduguri Staff Development and Study Fellowship grant and a Research Fellowship Award from the German Academic Exchange Service (DAAD) to Dr. M.I. Adah. The assistance of the technical staff of the Institute of Medical Microbiology and Virology, Ruhr University, Bochum, is appreciated.

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