Virology **274,** 309–320 (2000)
doi:10.1006/viro.2000.0456, available online at http://www.idealibrary.com on IDEAL®

Molecular and Serologic Characterization of Novel Serotype G8 Human Rotavirus Strains Detected in Blantyre, Malawi

Nigel A. Cunliffe,[1,2,7] Jon R. Gentsch,[3] Carl D. Kirkwood,[3] Jailosi S. Gondwe,[2] Winifred Dove,[7] Osamu Nakagomi,[4] Toyoko Nakagomi,[4] Yasutaka Hoshino,[5] Joseph S. Bresee,[3] Roger I. Glass,[3] Malcolm E. Molyneux,[1,6] and C. Anthony Hart[7,8]

[1]*Wellcome Trust Research Laboratories, Blantyre, Malawi;* [2]*Department of Paediatrics, College of Medicine, University of Malawi;* [3]*Viral Gastroenteritis Section, Centers for Disease Control and Prevention, Atlanta, Georgia 30333;* [4]*Department of Microbiology, Akita University School of Medicine, Akita, Japan;* [5]*Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892;* [6]*School of Tropical Medicine, University of Liverpool, Liverpool, United Kingdom; and* [7]*Department of Medical Microbiology and Genito-Urinary Medicine, University of Liverpool, United Kingdom*

Received March 7, 2000; returned to author for revision May 17, 2000; accepted June 1, 2000

During a 2-year study of diarrhea among children in Blantyre, Malawi, greater than 50% of rotavirus strains genotyped by using reverse transcription—polymerase chain reaction possessed previously unrecognized combinations of the neutralization proteins VP7 and VP4. Serotype G8 rotaviruses, which have been identified recently in several African countries, were found to possess P[4] or P[6] VP4 genotype specificity. Two of these short electropherotype rotaviruses were further investigated: these comprised a P[6], G8 representative strain (MW23) and a P[4], G8 representative strain (MW333). The VP7 gene sequences of both strains exhibited greatest homology to human and animal serotype G8 rotaviruses. Sequence analysis of the VP4 gene of MW23 indicated closest identity to the P2A[6], G9 strain US1205 from the United States. The VP4 gene of MW333 was most closely related to the P[4], G12 strain L26 isolated in the Philippines and the Australian P[4], G2 strain RV-5. The NSP4 gene sequences of both strains were classified in NSP4 genetic group I. RNA-RNA hybridization demonstrated that each of these two strains is related to the DS-1 genogroup of human rotaviruses. Subgroup analysis and virus neutralization confirmed complete antigenic characterization of MW23 as subgroup I, P2A[6], G8 and MW333 as subgroup I, P1B[4], G8. The similarity of the VP7 gene sequences of the prototype strains described in this report to bovine serotype G8 rotaviruses suggests that they may represent human/bovine reassortant viruses.

INTRODUCTION

Group A rotaviruses cause an estimated 870,000 deaths from acute, dehydrating gastroenteritis in children in developing countries each year (De Zoysa and Feachem, 1985). In industrialized countries, relatively few deaths can be directly attributed to rotavirus infection, but the morbidity and economic losses are substantial (Tucker *et al.,* 1998). In view of the global impact of rotaviruses, the development of effective rotavirus vaccines has long been considered a priority (Institute of Medicine, 1986). To this end, a number of vaccines have been produced, most of which have been designed to provide serotype-specific protection against the most prevalent rotavirus serotypes (Midthun and Kapikian, 1996). Four serotypes (G1 to G4), representing the most globally common rotavirus strains, were incorporated into a tetravalent vaccine that proved highly effective in the prevention of severe rotavirus diarrhea in field trials in the United States, Finland, and Venezuela (Rennels *et al.,* 1996; Joensuu *et al.,* 1997; Perez-Schael *et al.,* 1997).

In view of the serotype-specific protection thought necessary to confer maximum protection against rotavirus diarrhea, the formulation of effective rotavirus vaccines is critically dependent on knowledge of the diversity of circulating strains. Rotaviruses possess dual serotype specificity, determined by neutralizing antibody responses to the two outer capsid proteins, VP7 encoded by RNA segment 7, 8, or 9 and VP4 encoded by RNA segment 4. Fourteen VP7 (or G, for glycoprotein,) serotypes and 11 VP4 (or P, for protease-sensitive) serotypes are recognized (Estes, 1996; Okada *et al.,* 2000). Genetic characterization of VP4 genes indicates that there are at least 20 distinct P genotypes, including the genes corresponding to the 11 known P serotypes (Estes, 1996). Of these, 10 G serotypes and 8 P serotypes have been identified in humans.

Historically, rotavirus surveillance studies have been conducted using VP7-specific neutralizing monoclonal antibodies (N-MAbs) directed to the common human G serotypes (Taniguchi *et al.,* 1987). However, N-MAbs are only readily available to the common serotypes, and an increasing number of rare G types renders this form of

[8] To whom correspondence and reprint requests should be addressed at Department of Medical Microbiology and Genito-Urinary Medicine, University of Liverpool, Duncan Building, Daulby Street, Liverpool L69 3BX, UK. Fax: 44 151 706 5805. E-mail: cahmm@liv.ac.uk.

0042-6822/00 $35.00

typing less effective. Recently, molecular methods, especially reverse transcription-polymerase chain reaction (RT-PCR) and probe hybridization (Gouvea *et al.,* 1990; Gentsch *et al.,* 1992; Gunasena *et al.,* 1993; Flores *et al.,* 1990; Larralde and Flores, 1990), have been utilized in field studies to examine the genes encoding VP7 and VP4 (genotyping), which have served as proxies for serotype determination by N-MAbs. While an absolute correlation between serotype and genotype exists for VP7, the correlation for VP4 is less clear, and a complete description of VP4 requires both molecular and antigenic analysis. By convention, VP4 genotype is differentiated from VP4 serotype by placing the former within square brackets (Estes, 1996).

Numerous field studies of rotavirus strain diversity have documented that two main groups of strains predominate in many countries: G types 1, 3, and 4 possess P[8] VP4 specificity and serotype G2 strains carry the P[4] VP4 genotype (Gentsch *et al.,* 1996). These reports support earlier studies using liquid hybridization techniques, which suggested that reassortment between the two major human rotavirus genogroups (designated Wa and DS-1) was relatively uncommon (Flores *et al.,* 1985). However, more unusual G and P types, an expanding number of G/P type combinations, and reassortment between genogroups are now being reported with increasing frequency. For example, serotype G5 has been identified in Brazil (Gouvea *et al.,* 1994a; Leite *et al.,* 1996), and serotype G9 may be an emerging global serotype since recent reports have documented its presence in the United States (Ramachandran *et al.,* 1998), India (Ramachandran *et al.,* 1996), Bangladesh (Unicomb *et al.,* 1999), and Malawi (Cunliffe *et al.,* 1999). In the Bangladeshi study, five distinct G9 reassortants were characterized based on the identification of P and G type, subgroup, and electropherotype (Unicomb *et al.,* 1999). The VP4 P[6] genotype is also now recognized as an important P type in infants with diarrhea (Timenetsky *et al.,* 1994; Ramachandran *et al.,* 1996; Cunliffe *et al.,* 1999). Taken together, these data suggest that rotavirus strain diversity is much greater than previously appreciated.

In a recently completed 2-year study of viral gastroenteritis in children in Blantyre, Malawi, we identified a remarkable diversity of rotavirus strains (Cunliffe *et al.,* 1999). Serotype G8 rotaviruses were identified in 51% of rotaviruses detected between 1997 and 1998; the majority of these strains possessed P[6] VP4 specificity and some were of P[4] VP4 specificity. Our study was the first to describe serotype G8 as a predominant rotavirus strain; moreover, the combinations of P[6], G8 and P[4], G8 genotypes were previously unrecognized, suggesting that these strains were novel viral reassortants in respect to their surface neutralization proteins VP7 and VP4. Given the local importance of these two strains, together with recent reports of serotype G8 rotavirus in Africa and elsewhere (Adah *et al.,* 1997; Santos *et al.,* 1998; Holmes *et al.,* 1999; Nakata *et al.,* 1999; Steele *et al.,* 1999; Palombo *et al.,* 2000), we considered it important to examine these strains in more detail. Specifically, we sequenced the genes encoding the neutralization proteins VP7 and VP4, and the nonstructural protein NSP4, of a representative of the P[6], G8 genotype strains (designated MW23) and a representative of the P[4], G8 genotype strains (designated MW333). We also characterized the strains antigenically by subgrouping and virus neutralization, and examined the overall genetic relatedness of these strains to other human and animal strains by RNA-RNA hybridization (genogrouping).

RESULTS

Sequence analysis

The complete nucleotide (nt) and amino acid (aa) sequences of the gene segment encoding VP7 were determined for MW23 and MW333, and compared with the corresponding sequences of strains representing G types 1 to 14 (Table 1). The VP7 gene of both strains was 1062 nt in length. A single open-reading frame (ORF) extended from nt 49 to nt 1026, encoding a protein of 326 aa in length. Sequence comparisons (Table 1) and phylogenetic analysis (Fig. 1) indicated that the VP7 sequences of MW23 and MW333 were most closely related to human and animal serotype G8 rotaviruses. Both strains exhibited closest identity with the VP7 gene of the Nigerian serotype G8 strain HMG89 (>97% nt and aa identities), and the closest identities with VP7 genes of animal rotaviruses were with bovine strain A5 (93.6% aa identities for both strains). Rotavirus strains representing other G serotypes exhibited much less nucleotide and amino acid homology (<82%) with MW23 and MW333.

Complete gene 4 sequence, encoding VP4, was determined for MW23 and MW333 and compared with strains representing various P types (Table 2). Gene 4 of both strains was 2359 nt in length. The ORF extended from nt 10 to nt 2334, encoding a protein of 775 aa. The VP4 sequence of MW23 showed closest homology to rotaviruses of P[6] VP4 genotype, with greatest identity to the recently characterized United States P2A[6], G9 strain US1205 (97.1% nt and 97.5% aa identities). The VP4 sequence of MW333 displayed closest homology to rotaviruses of P[4] VP4 genotype and was most closely related to Filipino P[4], G12 strain L26 (95.3% nt and 96.1% aa identities) and Australian P[4], G2 strain RV-5 (94.1% nt and 95.9% aa identities). Phylogenetic analysis confirmed the VP4 assignments (Fig. 2).

A 739-bp fragment of gene 10 was sequenced for each strain, encoding the nonstructural protein, NSP4. The ORF for both strains extended from nt 42 to nt 566, encoding a protein of 175 aa. Both strains showed greatest nucleotide and amino acid NSP4 homologies with genetic group I (DS-1-like) viruses (Table 3).

TABLE 1

Comparison of the Gene Encoding VP7 of Malawi Rotavirus Strains MW23 and MW333 with Other Human and Animal VP7 Genes

Strain[a]	Species of origin	G type	MW23		MW333	
			nt identity (%)	aa identity (%)	nt identity (%)	aa identity (%)
MW23	**Human**	**8**	—	—	**98.0**	**97.9**
MW333	**Human**	**8**	**98.0**	**97.9**	—	—
HMG89	Human	8	97.6	97.2	97.8	97.9
EGY1850	Human	8	89.1	95.1	89.3	95.1
HAL1166	Human	8	83.8	94.2	84.4	94.2
QEH14262	Human	8	87.5	93.6	87.9	93.3
GR570/85	Human	8	91.1	93.9	91.2	93.9
DG8	Human	8	84.0	92.9	84.7	92.9
69M	Human	8	84.2	92.9	84.9	92.9
B37	Human	8	83.1	90.5	83.6	90.5
A5	Bovine	8	86.4	93.6	86.9	93.6
Cody-I801	Bovine	8	83.0	92.3	83.5	92.3
Wa	Human	1	72.2	77.3	72.1	77.6
S2	Human	2	71.9	73.6	71.9	73.9
HCR3	Human	3	76.3	81.9	76.5	81.6
HOCHI	Human	4	71.9	72.4	72.0	72.4
OSU	Porcine	5	75.5	80.7	75.3	80.1
UK	Bovine	6	74.3	81.0	74.8	81.3
116E	Human	9	75.6	78.5	75.4	78.2
I321	Human	10	73.7	78.8	73.9	78.8
YM	Porcine	11	75.0	81.3	74.8	80.7
L26	Human	12	74.2	76.1	74.8	76.7
L338	Equine	13	72.7	75.2	73.0	75.5
FI23	Equine	14	72.8	78.2	73.1	78.2

[a] The accession numbers of the reference sequences were: HMG89, X98918; EGY1850, AF104102; HAL1166, L20882; QEH14262, AF143689; GR570/85, AF143688; DG8, AF034852; B37, J04334; A5, D01054; Cody-I801, U14999; Wa, K02033; S2, M11164; HCR3, L21666; HOCHI, ABO12078; OSU, X04613; UK, X00896; 116E, L14072; I321, L07658; YM, M23194; L26, M58290; L338, D13549; FI23, M61876. The nt sequence of strain 69M was obtained from Green *et al.* (1989).

Antigenic characterization

Subgroup analysis of culture-adapted strains MW23 and MW333 was performed by enzyme immunoassay. Both strains were of subgroup I specificity (data not shown).

The P serotypes of MW23 and MW333 were determined by plaque reduction neutralization assays using hyperimmune serum raised to five reassortant rotavirus strains, each representing a different P serotype (Table 4). Strain MW23 was neutralized to a significant level only by hyperimmune antisera raised against strain ST3 × DS-1 (P2A[6], G2), indicating that MW23 belongs to serotype P2A. Strain MW333 was neutralized to a significant level only by antisera raised against strain S2 × UK (P1B[4], G6), indicating that MW333 belongs to serotype P1B. Further antigenic analysis of the VP4 protein of MW23 was undertaken with neutralization experiments using the three VP4-specific N-MAbs HS-6, HS-16, and F45:4. The neutralization results were compared with those obtained by Kirkwood *et al.* (1999) for the genetically closely related strain US1205 and standard strains M37, ST3, and F45 (Table 5). In contrast to strains M37 and ST3, neither MW23 nor US1205 was neutralized with HS-6 or HS-16. Furthermore, the P1A, P2A cross-reactive N-MAb F45:4 neutralized MW23 and US1205 to low titer compared to the much higher titers obtained with M37 and F45. Thus, MW23 and US1205 share similar VP4 antigenic profiles, which differ from the standard P2A[6] strains M37 and ST3.

A single VP7-specific N-MAb (B37:1) raised against the serotype G8 strain B37 neutralized MW23 to high titer, indicating that MW23 belongs to serotype G8 (data not shown).

Genogroup analysis

The standard short electropherotype profiles of strains MW23 and MW333 suggested a relationship to DS-1 genogroup strains. To investigate this, we used probes derived from the standard human strains Wa and DS-1, representing the Wa genogroup and the DS-1 genogroup, respectively (Fig. 3A), and from Malawi strains MW23 (P[6], G8) (Fig. 3B) and MW333 (P[4], G8) (Fig. 3C). Analysis of MW23, MW333, and several other related Malawi genotype P[6], G8 strains confirmed a strong relationship with the DS-1 genogroup; 7–9 strong hybrid bands (corresponding to segments 2–10) were observed

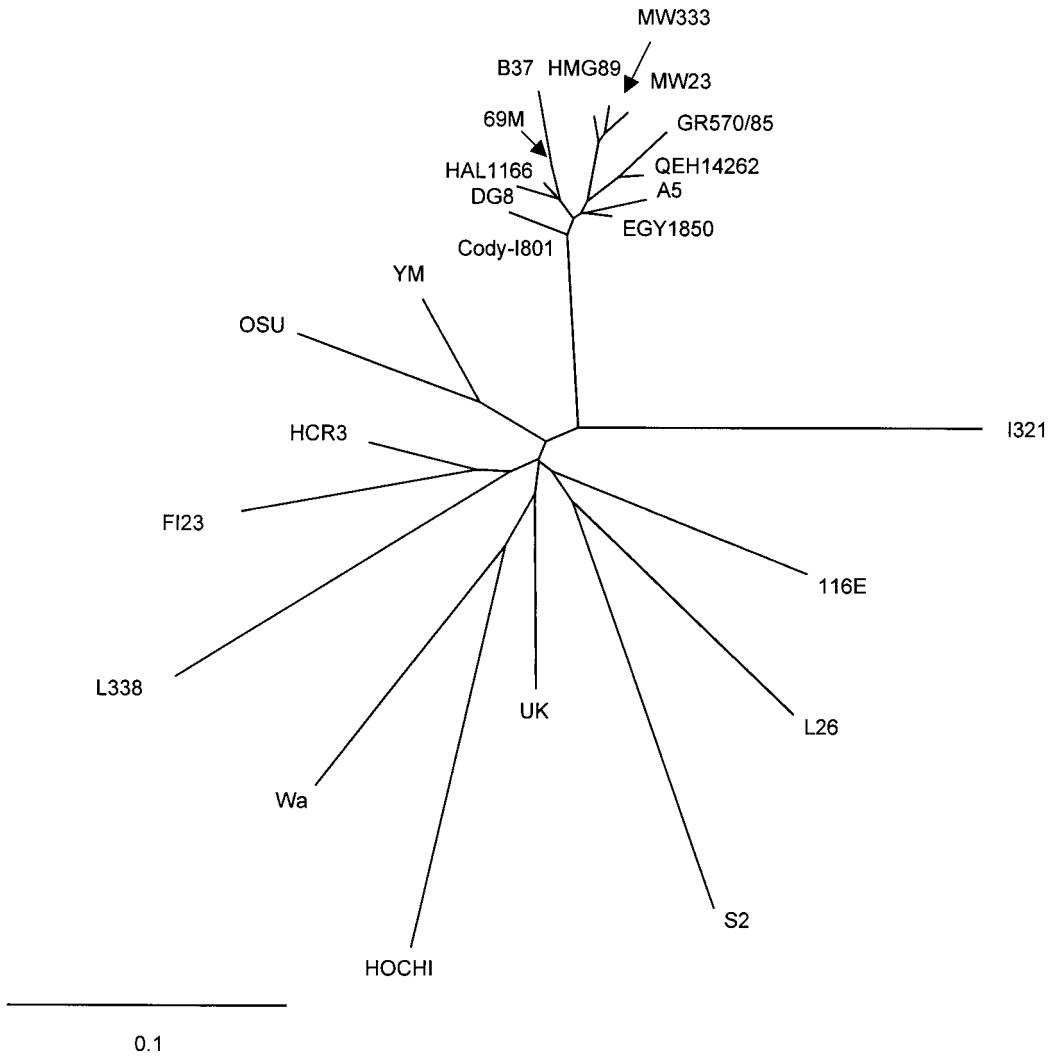

FIG. 1. Neighbor-joining protein distance tree for amino acid residues representing complete VP7 of the indicated strains. Sequences were aligned by using CLUSTALX and analyzed by using the PROTDIST and NEIGHBOR programs in PHYLIP.

between the DS-1 probe and the Malawi strains. In contrast, only 1–2 weak hybrid bands were observed between the Wa probe and the Malawi strains. In the reciprocal experiments, probes from Malawi strains MW23 and MW333 formed 7–11 hybrid bands with each other and 6–7 weaker hybrid bands with strain DS-1 but formed 0–1 faint hybrids with Wa and AU-1 strains. Thus, this experiment confirmed the genetic relatedness of these strains to the DS-1 genogroup but suggested that divergence has occurred between the Malawi strains and prototype DS-1. In addition, different hybridization patterns were noted between the Malawi strains; divergence between MW23-like and MW333-like strains was observed not only in the expected difference between their VP4 genes but also in other high and low-molecular-weight RNA bands. Finally, this experiment confirmed the relationship of DS-1 genogroup strains such as MW23 and MW333 to the P4[10], G8 strain 69M, which has been previously documented (Ohshima *et al.,* 1990). A reciprocal experiment confirmed that strain 69M probe gave a similar pattern of binding to 3 of 4 Malawi strains (data not shown).

DISCUSSION

Because serotype G8 appears to be the most common rotavirus serotype in Blantyre, Malawi, we characterized two serotype G8 rotavirus strains, MW23 and MW333, that were recently detected in stool specimens of children with diarrhea. These short electropherotype rotaviruses were characterized by using RT-PCR analysis of the VP7 and VP4 genes as genotypes P[6], G8 (MW23) and P[4], G8 (MW333). When we used a combination of nucleotide sequence analysis, subgrouping, and virus neutralization, MW23 was confirmed as subgroup I, serotype P2A[6], G8 and MW333 was characterized as subgroup I, serotype P1B[4], G8. RNA-RNA hybridization

TABLE 2

Comparison of the Gene Encoding VP4 of Malawi Rotavirus Strains MW23 and MW333 with Other Human and Animal VP4 Genes

Strain[a]	Species of origin	P type	MW23		MW333	
			nt identity (%)	aa identity (%)	nt identity (%)	aa identity (%)
MW333	**Human**	**4**	**73.8**	**77.0**	—	—
L26	Human	4	74.3	77.3	95.3	96.1
RV-5	Human	4	74.0	76.8	94.1	95.9
MW23	**Human**	**6**	—	—	**73.8**	**77.0**
US1205	Human	6	97.1	97.5	73.9	77.7
RV-3	Human	6	95.7	96.5	73.7	77.2
M37	Human	6	95.3	96.1	73.4	77.7
ST3	Human	6	95.5	95.0	73.8	77.0
1076	Human	6	94.8	94.3	73.4	75.7
GOTTFRIED	Porcine	6	82.7	89.2	74.9	77.0
A5	Bovine	1	70.0	73.6	69.9	72.4
SA11	Simian	2	71.6	73.6	70.9	72.0
RRV	Simian	3	71.1	74.2	68.9	72.7
UK	Bovine	5	67.4	70.3	67.4	68.9
OSU	Porcine	7	69.4	71.4	69.4	70.4
Wa	Human	8	75.0	78.3	87.1	89.0
AU-1	Human	9	64.9	66.1	66.1	65.5
69M	Human	10	70.5	74.5	70.8	72.4
116E	Human	11	59.7	58.1	61.3	60.3
H2	Equine	12	70.4	74.5	71.4	71.7
MDR-13	Porcine	13	68.6	70.5	68.9	69.9
HAL1166	Human	14	65.8	66.2	64.5	66.8
Lp14	Ovine	15	71.0	74.3	69.9	71.7
Eb	Murine	16	65.1	67.7	66.0	68.0
993/83	Bovine	17	62.4	61.5	62.4	60.2
L338	Equine	18	71.4	73.6	70.5	70.4
4F	Porcine	19	75.5	82.3	75.4	79.1
EHP	Murine	20	68.2	72.5	66.5	71.6

[a] The accession numbers of the reference sequences were L26, M36397; RV-5, M32559; US1205, AF079356; RV-3, U16299; M37, L20877; ST3, L33895; 1076, M88480; GOTTFRIED, M33516; A5, D13395; SA11, D16346; RRV, M18736; UK, M22306; OSU, X13190; Wa, L34161; AU-1, D10970; 69M, M60600; 116E, L07934; H2, L04638; MDR-13, L07886; HAL1166, L20875; Lp14, L11599; Eb, L18992; 993/83, D16352; L338, L26888; 4F, L10359; EHP, U08424.

demonstrated that both strains were related to the DS-1 genogroup of human rotaviruses.

The VP7 sequences of both strains were most closely related to known serotype G8 rotaviruses of human and bovine origin; their closest relationships were to each other and to Nigerian strain HMG89 (Adah *et al.,* 1997) (>97% aa identity), strongly suggesting that the Malawi strains belong to serotype G8. The ability of the MAb B37:1 to neutralize MW23 strongly supports the sequence data and confirms their serotype assignment as G8. Both MW23 and MW333 were also quite closely related to serotype G8 human rotaviruses detected in Egypt (EGY1850; 95.1% aa identity) and South Africa (GR570/85; 93.9% aa identity), raising the possibility of a common lineage for the African serotype G8 strains. Phylogenetic analysis, indicating some clustering of the African strains, lends support to this hypothesis (Fig. 1). The VP7 sequence relationships of both strains to bovine rotaviruses A5 (93.6% aa identity) and Cody (92.3% aa identity) confirm the well-reported similarity between human and bovine VP7 genes (Gerna *et al.,* 1994).

Nucleotide sequencing of the VP4 gene of MW23, and neutralization using hyperimmune serum to different single VP4 gene reassortant rotaviruses, confirmed that MW23 belongs to P serotype P2A and genotype P[6]. The VP4 sequence relatedness (97.5% aa identity) of MW23 to the P2A[6], G9 strain US1205 described by Kirkwood *et al.* (1999) was also reflected in similar antigenic profiles using the VP4-specific N-MAbs HS-6, HS-16, and F45:4. Sequence analysis of MW333 and P-serotyping studies confirmed its VP4 antigenic type as P1B[4]. The closest related published strains were the Filipino P[4], G12 strain L26 described by Taniguchi *et al.* (1990) and P[4], G2 strain RV-5 isolated in Australia (Kantharidis *et al.,* 1987).

Sequence analysis of a 739-bp gene 10 fragment of strains MW23 and MW333 confirmed their expected classification in NSP4 genetic group I, which comprises DS-1 like rotaviruses (Cunliffe *et al.,* 1997). As for its VP4 gene, the sequence of MW23 NSP4 was very closely related (99.4% aa identity) to the United States strains US1205 and US1261, and recent Australian isolate E210.

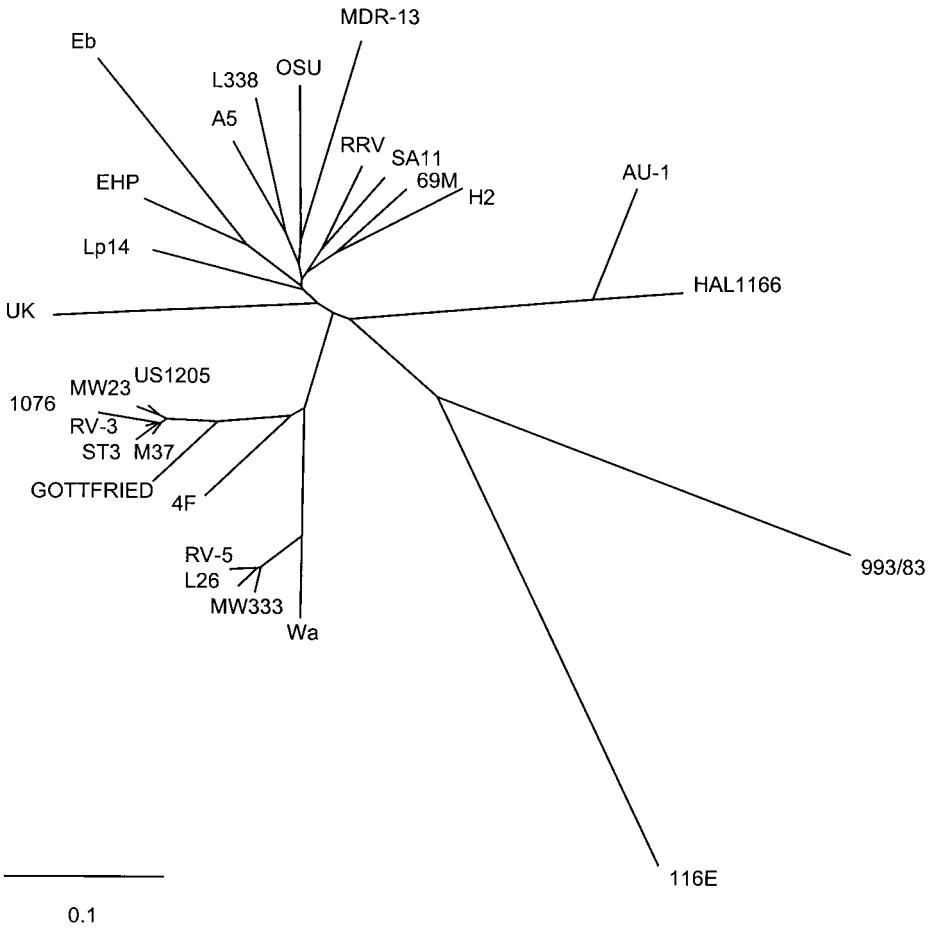

FIG. 2. Neighbor-joining protein distance tree for amino acid residues representing complete VP4 of the indicated strains. Sequences were aligned by using CLUSTALX and analyzed by using the PROTDIST and NEIGHBOR programs in PHYLIP.

In contrast, the MW333 NSP4 sequence was slightly more divergent (96.0% aa identity to MW23 and 96.6% aa identity to US1205, US1261, E210, and bovine strain UK). Together, these sequence data support an unusually close relationship between P2A[6], G9 strains isolated in the United States and P2A[6], G8 strains from Malawi.

RNA-RNA hybridization confirmed that both MW23 and MW333 belong to the DS-1 genogroup. Detectable differences between MW23-like (P2A[6], G8) and MW333-like (P1B[4], G8) strains were evident; the observed differences in hybridization patterns of specific lower and higher molecular weight RNA segments suggest that these genes could have originated from differing sources. The close homology of MW23 VP4 and NSP4 genes, and of the MW333 NSP4 gene, with United States P2A[6], G9 strains identified by sequence analysis raises questions on the overall homology between these recently isolated strains.

The novel combinations of the VP7 and VP4 proteins of MW23 and MW333 are most likely to have arisen by reassortment between the corresponding genome segments of two distinct rotaviruses. Rotavirus gene reassortment occurs readily *in vitro* (Graham *et al.,* 1987) and is likely to be an important mechanism whereby rotaviruses evolve (Nakagomi and Nakagomi, 1993; Taniguchi and Urasawa, 1995). Typical short electropherotype, DS-1-like P[4] rotaviruses possess serotype G2 VP7 specificity: the P[4], G8 strains detected in Malawi (prototype strain MW333) may therefore represent single VP7 gene substitution reassortants, perhaps resulting from a dual infection of a DS-1-like P[4], G2 rotavirus and a serotype G8 rotavirus. Similarly, the P[6], G8 strains (prototype MW23) may possibly have formed as a result of reassortment between a G8 rotavirus and a short electropherotype P[6] strain, e.g., a P[6], G9 strain from Malawi (Cunliffe *et al.,* 1999). This raises the possibility that MW23 is a multigene reassortant between Wa and DS-1 genogroups. While *in vitro* experiments suggest that reassortment across genogroups is restricted (Ward and Knowlton, 1989), evidence is growing that intergenogroup reassortants may occur and persist in nature more frequently than expected (Ward *et al.,* 1990; Krishnan *et al.,* 1994; Unicomb *et al.,* 1999; Ramachandran *et al.,* unpublished). The failure to detect serotype G2 rotaviruses in Malawi in surveillance between 1997 and 1999 (Cunliffe *et al.,* unpublished) allows us to speculate that

TABLE 3

Comparison of the Gene Encoding NSP4 of Malawi Rotavirus Strains MW23 and MW333 with Other Human and Animal NSP4 Genes

Strain[a]	Species of origin	Genetic group	MW23		MW333	
			nt identity (%)	aa identity (%)	nt identity (%)	aa identity (%)
MW23	**Human**	**I**	—	—	**93.8**	**96.0**
MW333	**Human**	**I**	**93.8**	**96.0**	—	—
US1261	Human	I	99.3	99.4	94.2	96.6
US1205	Human	I	99.1	99.4	93.9	96.6
E210	Human	I	97.4	99.4	93.9	96.6
KUN	Human	I	96.1	98.9	94.7	96.0
S2	Human	I	97.2	98.3	94.2	95.4
RV-5	Human	I	94.6	94.3	94.3	94.3
1076	Human	I	91.5	92.6	91.6	92.6
UK	Bovine	I	93.0	96.6	92.7	96.6
NCDV	Bovine	I	88.5	94.3	89.6	94.3
SA11	Simian	I	88.6	93.1	91.3	94.3
RV-4	Human	II	83.1	84.6	84.0	82.9
Wa	Human	II	83.1	84.6	83.7	82.9
M37	Human	II	83.9	84.6	84.3	82.9
YM	Porcine	II	81.3	83.4	82.5	82.9
ST3	Human	II	82.5	82.3	83.5	80.6
AU-1	Human	III	82.7	84.0	82.7	83.4
RRV	Simian	III	81.7	83.4	82.2	82.9

[a] The accession numbers of the reference sequences were US1261, AJ236751; US1205, AF079358; E210, U59107; KUN, D88829; S2, U59104; RV-5, U59103; 1076, U59105; UK, K03384; NCDV, X06806; SA11, K01138; RV-4, U59108; Wa, K02032; M37, U59109; YM, X69485; ST3, U59110; AU-1, D89873; RRV, L41247.

the formation of these strains may potentially mark an evolutionary change in the genetic make-up of DS-1-like rotaviruses.

The sequence homology between the VP7 genes of MW23 and MW333, and serotype G8 rotaviruses of bovine origin, prompts speculation that the VP7 genes of the prototype strains described in this report may have originated from a bovine source. The possibility of interspecies transmission of rotaviruses, either as whole virions or by genetic reassortment, was reviewed by Nakagomi and Nakagomi (1993). Serotype G8 rotaviruses are well-known pathogens in cattle (Snodgrass *et al.,* 1990; Taniguchi *et al.,* 1991; Sato *et al.,* 1997) and have also been detected in pigs (Gouvea *et al.,* 1994b) and a horse (Isa *et al.,* 1996). Initially suggested by Taniguchi *et al.* (1990), the possibility of interspecies transmission of VP7 genes between cattle and humans has frequently been considered (Ohshima *et al.,* 1990; Taniguchi *et al.,* 1991; Browning *et al.,* 1992; Gerna *et al.,* 1994; Sato *et al.,* 1997; Holmes *et al.,* 1999; Palombo *et al.,* 2000). The sharing of human and bovine VP4 genes was suggested for the bovine-like P[11] VP4 gene of Indian neonatal strain 116E (Gentsch *et al.,* 1993), and the recent identification of this VP4 type in cow and buffalo calves in India strengthens this hypothesis (Gulati *et al.,* 1999). The transfer of VP7 genes, or whole virions, between humans and cattle is

TABLE 4

Reciprocal of 60% Fluorescent Focus Neutralization Antibody Titer of Guinea Pig Hyperimmune Antiserum to Indicated Reassortant for Strains MW23 and MW333

Hyperimmune antiserum to reassortant	Virus strain		
	MW23	MW333	
Wa × DS-1 (P1A[8], G2)	160	80	(2560)[a]
S2 × UK (P1B[4], G6)	<80	1280	(5120)[a]
ST3 × DS-1 (P2A[6], G2)	2560	80	(2560)[a]
K8 × DS-1 (P3[9], G2)	<80	<80	(2560)[a]
69M × DS-1 (P4[10], G2)	320	80	(10240)[a]

[a] Reciprocal of 60% fluorescent focus neutralization antibody titer to VP4-homologous virus (e.g., Wa virus vs anti-Wa × DS-1, 1:2560)

TABLE 5

Neutralization Resistance Pattern of MW23 and US1205 by VP4-Specific N-MAbs Compared to Standard Strains

Virus strain	N-MAb		
	HS-6 (aa 72)	HS-16	F45:4 (aa 392)
MW23	**410**	**<200**	**3,500**
US1205[a]	780	580	2,600
ST3[a]	17,000	170,000	2,400
M37[a]	2,300	160,000	10,000
F45[a]	810	<100	51,000

[a] Data from Kirkwood *et al.* (1999).

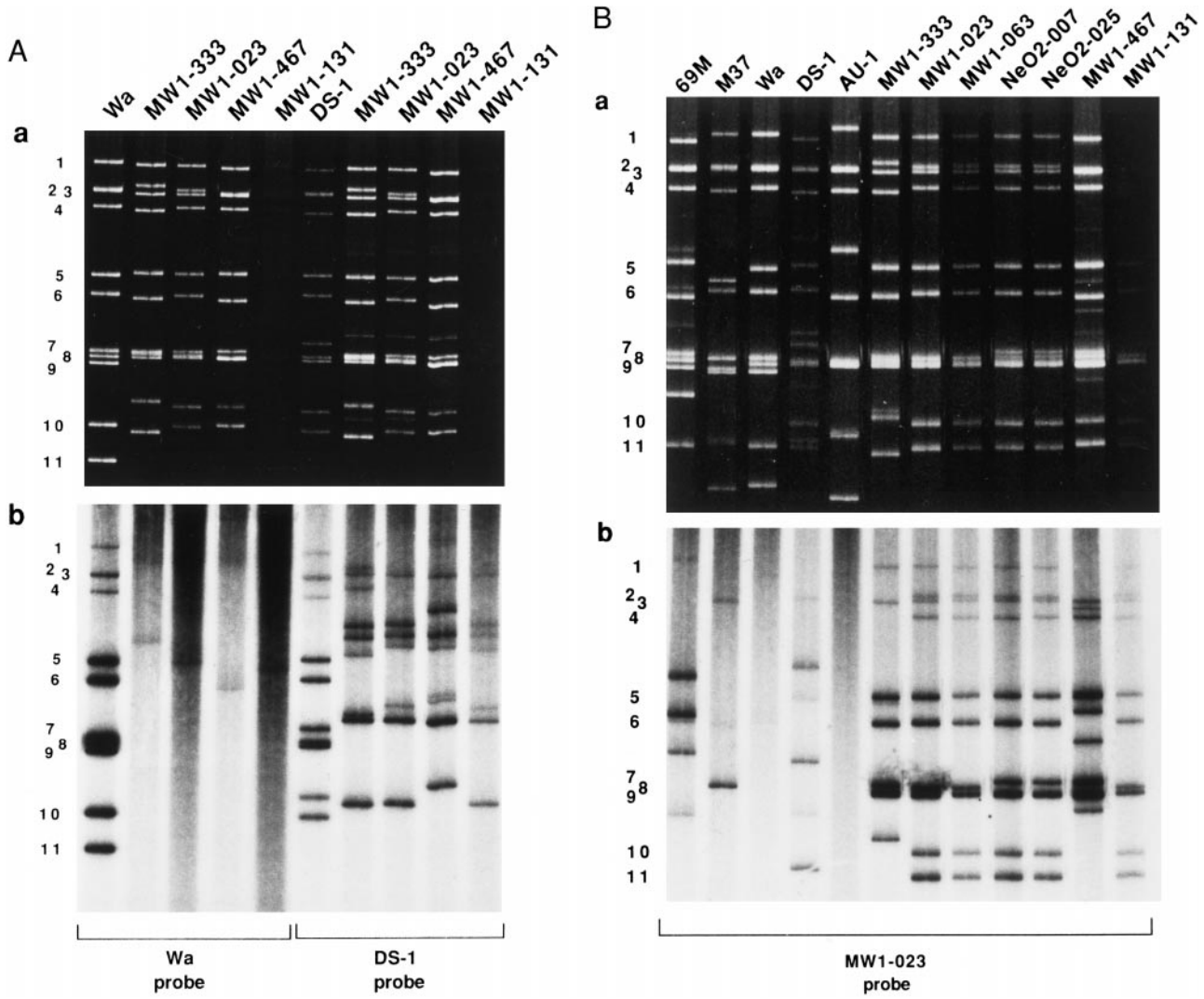

FIG. 3. (A–C) Hybridization patterns between genomic RNAs from the indicated virus strains and the 32P-labeled, plus-strand transcription probes prepared from strains Wa and DS-1 (A), MW1-023 (B), and MW1-333 (C). Ethidium bromide-stained gels under UV light illumination (panel a) revealed genomic RNAs from the indicated strain. Faint bands represent aberrantly migrating hybrid bands formed between the probe and the genomic RNAs and correspond to the hybrid bands appearing on the autoradiograph (panel b). Approximate positions of the RNA segments of strains Wa (A), 69M (B), and MW1-333 (C) are indicated to the left. Throughout this manuscript, strain MW1-023 is referred to as MW23, and strain MW1-333 is referred to as MW333.

theoretically possible in Malawi, where in some regions they live in close proximity. It will be of interest to search for these novel strains in animal reservoirs in Malawi.

Since the first detection of serotype G8 human rotaviruses in Indonesia (Hasegawa *et al.,* 1984; Matsuno *et al.,* 1985; Albert *et al.,* 1987), reports have documented the presence of this serotype in Europe (Gerna *et al.,* 1990; Beards and Graham, 1995; Steele *et al.*); Brazil (Santos *et al.,* 1998), and Australia (Palombo *et al.,* 2000). Importantly, several reports of serotype G8 rotaviruses in African countries have now been published (Adah *et al.,* 1997; Cunliffe *et al.,* 1999; Holmes *et al.,* 1999; Nakata *et al.,* 1999; Steele *et al.,* 1999). It is not known whether current reassortant rotavirus vaccines (that do not include the VP7 gene of a serotype G8 rotavirus) will afford protection against serotype G8 strains. This is an important question, since G8 may be an emerging serotype globally, and especially in African countries, where the need for effective rotavirus immunization is great (Cunliffe *et al.,* 1998).

MATERIALS AND METHODS

Rotavirus strains

Human rotaviruses characterized as P[6], G8 and P[4], G8 were detected by enzyme immunoassay (Rotaclone, Meridian Diagnostics, Cincinnati, OH) in stool specimens obtained from children hospitalized with rotavirus diarrhea in Blantyre, Malawi. Strains MW23 (detected in the stool of an 8-month-old infant) and MW333 (detected

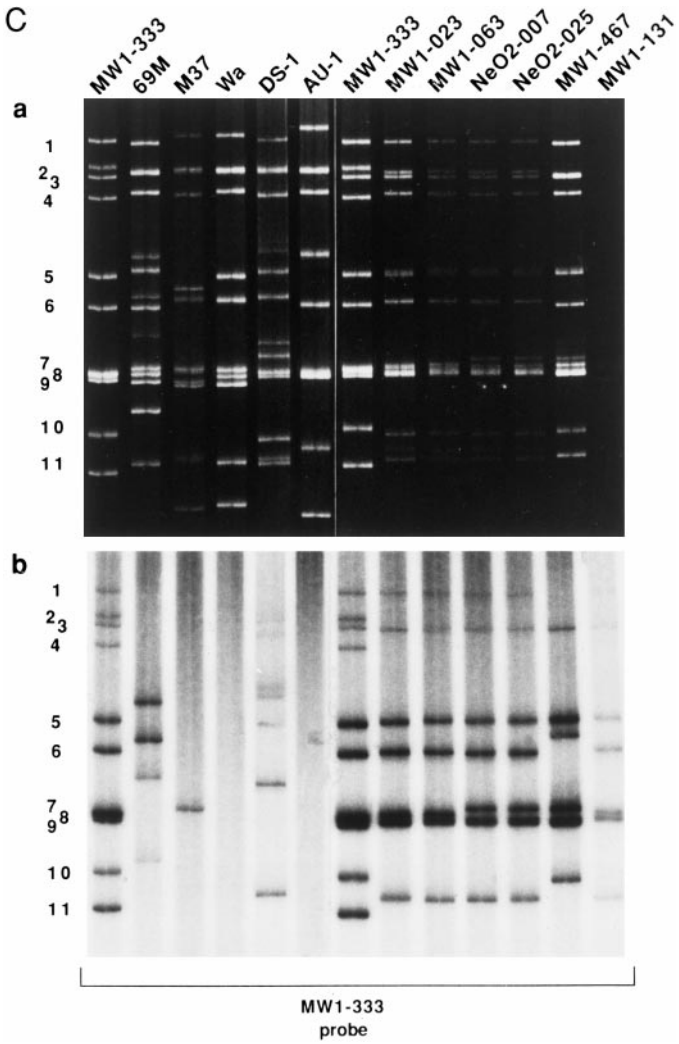

FIG. 3—*Continued*

in the stool of a 6-month-old infant) were selected for more detailed characterization as representative members of their respective genotypes on the basis of their electrophoretic mobilities on a 10% polyacrylamide gel. The strains were adapted to culture in monkey kidney (MA104) cells in the presence of trypsin.

Strain B37 (serotype G8) was used in N-MAb studies. Viral reassortants used in neutralization experiments were Wa × DS-1 (P1A[8], G2), S2 × UK (P1B[4], G6), ST3 × DS-1 (P2A[6], G2), K8 × DS-1 (P3[9], G2), and 69M × DS-1 (P4[10], G2). Strains Wa (P1A[8], G1), DS-1 (P1B[4], G2), AU-1 (P3[9], G3), 69M (P4[10], G8), and M37 (P2A[6], G1) were used in RNA hybridization studies. Strains DS-1 (subgroup I) and Wa (subgroup II) were used in subgrouping experiments.

Reverse transcription-polymerase chain reaction

Rotavirus dsRNA was obtained from infected cell cultures by using phenol/chloroform extraction and ethanol precipitation followed by partial purification with a guanidine and silica method (Gentsch *et al.,* 1992). The dsRNA gene segments 9, 4, and 10 encoding proteins VP7, VP4, and NSP4, respectively, were denatured, reverse transcribed, and then amplified by PCR. Using degenerate primer pair beg9 and end9, full-length gene 9 was obtained for both strains (Gouvea *et al.,* 1990). For gene 4, primers con2 and con3 were used to amplify an 877-bp fragment covering the VP8* region of both MW23 and MW333 (Gentsch *et al.,* 1992). The 5′ terminal gene 4 sequences of both strains were amplified by using negative-sense primers designed from MW23 and MW333 sequence, in RT-PCR together with primers designed from the respective published 5′ terminal sequences of strain RV3 (Kirkwood *et al.,* 1996) and L26 (Taniguchi *et al.,* 1990). The VP5* fragment of MW23 was amplified by using a primer complementary to con2 and the 3′ end primer NnendG4 described by Kirkwood *et al.* (1999). For MW333, the VP5* fragment was amplified as a 1496-bp product (encompassing bases 864–2359) using primers jrg124 (5′-att ggg ata taa atg gtc tga aat-3′) and jrg125 (5′-ggt cac atc ctc gat gac att-3′). A 739-bp fragment of the NSP4 gene 10 was amplified by using primers described previously (Cunliffe *et al.,* 1997).

Nucleotide sequencing

Complementary DNA obtained for each gene was purified by using gel extraction or spin columns (Qiagen, Chatsworth, CA). For VP7, since weak beg9/end9 product gave poor sequence when used directly, the purified beg9/end9 products of both strains were first cloned into a PGEMT plasmid vector and then sequenced by using universal and reverse primers in addition to VP7-specific primers. For VP4 and NSP4, purified PCR products were sequenced directly with VP4- and NSP4-specific primers. Each gene or gene fragment was sequenced completely in both directions by using the PRISM ready big dye terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, CA) with an automated sequencer (Applied Biosystems Model 377). Sequence analysis was performed by using the Sequencher program (Gene Codes Corp., Inc., Ann Arbor, MI) and the Wisconsin Genetics Computer Group computer programs (Devereux *et al.,* 1984; Felsenstein, 1989). Sequences were aligned with DNASTAR (Madison, WI), and phylogenetic trees were drawn by using PHYLIP version 3.5c (copyright J. Felsenstein and the University of Washington) and CLUSTALX (EMBL, Heidelberg, Germany).

The nucleotide sequences of strains MW23 and MW333 have been submitted to the EMBL Nucleotide Sequence Database. The accession numbers of the NSP4, VP4, and VP7 nucleotide sequences for strain MW23 are respectively AJ278252, AJ278253, and AJ278254; and for strain MW333 are respectively AJ278255, AJ278256, and AJ278257.

Subgroup analysis

Subgroup determination was performed by using subgroup I- and subgroup II-specific MAbs in an enzyme immunoassay method that has been previously described (Greenberg *et al.,* 1993).

Neutralization assays

VP4 serotype determination was performed by a 60% fluorescent focus neutralization assay that used hyperimmune antiserum prepared by intramuscular injection of guinea pigs to single VP4 gene reassortants (Hoshino *et al.,* 1984).

Antigenic analysis of VP7, and further antigenic analysis of VP4, utilized VP7- and VP4-specific N-MAbs that employed an immunofluorescent technique with MA104 cells grown in 96-well microtiter trays (Coulson *et al.,* 1985). The VP7-specific N-MAb B37:1 used in this study has been described previously (Tursi *et al.,* 1987). The VP4-specific N-MAbs HS-6, HS-16, and F45 were described by Padilla-Noriega *et al.* (1993) and Kirkwood *et al.* (1996).

Genogroup analysis

RNA-RNA hybridization was performed as previously described (Nakagomi *et al.,* 1989). Briefly, the 32P-labeled, single-stranded RNA probes from strains Wa, DS-1, MW23, and MW333 were hybridized to the denatured genomic RNAs from a panel of human rotavirus strains. Hybridization was allowed to occur at 65°C for 16 h in a buffer containing 25 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, and 0.1% sodium dodecyl sulfate (pH 8.0). The resulting hybrids were then precipitated with ethanol and were separated on a 10% polyacrylamide gel. Reannealed genomic RNAs were visualized by staining with ethidium bromide under UV illumination. Autoradiographs were prepared by exposing dried gels to BioMax MS films (Eastman Kodak Co., Rochester, NY).

ACKNOWLEDGMENTS

Nigel Cunliffe is a Wellcome Research Training Fellow in Clinical Tropical Medicine (Grant 049485/Z/96). We thank Professor Robin Broadhead and the staff of the Department of Paediatrics, College of Medicine, for their support during the course of this work; Ida Nkhonjera and Constance Magola for collecting samples; and the parents and guardians of the children involved in this study for their willing participation. We thank Reietsu Ito for technical assistance.

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