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Molecular epidemiology of human rotaviruses in Manipur: genome analysis of rotaviruses of long electropherotype and subgroup I

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Summary. In 1987/88 a winter outbreak of infantile gastroenteritis occurred in Manipur, India which was mainly due to rotaviruses of long electropherotype and subgroup (SG) I. The VP7 gene of one of these viruses (M48) has been cloned and sequenced. It was found to be very closely related to the VP7 genes of the G2 serotype human rotaviruses RV-5 and S2. Follow-up epidemiology of this event in Manipur during 1989–1992 yielded mainly rotaviruses of more conventional characteristics (94 isolates of SG II and long electropherotype, and 90 isolates of SG I and short electropherotype), but also 6 isolates of SG I with long electropherotype, indicating that these viruses continue to circulate in the Manipur community. One isolate of short electropherotype was of subgroup II, and one long electropherotype isolate reacted with the group A but not with either the subgroup I or subgroup II monoclonal antibodies.

Introduction

Rotaviruses are the major viral cause of acute gastroenteritis in infants and young children and in the young of many animal species. Rotaviruses consist of at least five groups (A–E). Group A is further subdivided into at least 4 subgroups (I, II, I & II, non-I, non-II) and 14 VP7-specific serotypes (G1–G14). Some serotypes have only been found in man (serotypes G9 and G12) and others only in animals (serotypes G5, G7, G11, G13, and G14), but several are detectable in both humans and animals (serotypes G1, G2, G3, G4, G6, G8 and G10) [4, 5, 6, 11, 13, 15, 19, 20, 31, 50, 51].

In 1989, Ghosh and Naik [21] described an outbreak of infantile gastroenteritis which had occurred in Manipur, India, during the winter of 1988/89. The interesting feature was that most isolates ($39/58 = 67.2\%$) were of long

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electropherotype and subgroup I. Normally human rotaviruses are of long electropherotype and subgroup II (mostly G1, G3 and G4 serotypes) or of short electropherotype and subgroup I (serotype G2). Most animal isolates of long electropherotype are of subgroup I. Therefore, this human outbreak was suspected to have originated from an animal reservoir, and it was of interest to investigate these viruses in more detail.

Here we report the nucleotide and predicted amino acid sequences of the serotype specifying VP7 gene of one of these isolates and compare them with sequences of corresponding genes of other group A rotaviruses. Some epidemiological data of rotavirus infections in Manipur in the years following the outbreak described by Ghosh and Naik [21] are also described.

Materials and methods

Viruses

The specimens from the study of Ghosh and Naik [21] were used. One of them with a high virion RNA content (M48) was chosen for cloning and sequencing.

A total of 1397 faecal specimens were obtained between 1989 and 1992 from 2 different sites in Manipur, India: (1) field sites comprising one surburban and two urban areas in Imphal, Manipur, with families having children below five years of age and rearing different domestic livestock and (2) three hospitals in Manipur (most isolates were from the Regional Medical College Hospital in Imphal, a few isolates from the Jawaharlal Nehru Hospital in Imphal, and five isolates from the District Hospital in Bishenpur). 787 faecal specimens from diarrhoea cases, 153 specimens from their contacts and 457 specimens of non-diarrhoeic controls (matched controls in field study, and children without diarrhoea undergoing treatment for other diseases in hospitals) were screened for rotaviruses during the epidemiological survey. Faecal specimens collected by the survey team in Manipur were transferred to the National Institute of Cholera & Enteric Diseases, Calcutta, for further analysis. The epidemiological survey also included screening of 332 faecal specimens from various animals (numbers screened for rotaviruses): monkeys (161), chicken (154), pigs (7), cows (4), ducks (4), pigeons (1) and cat (1). Forty three faeces were from animal contacts in households with diarrhoeic children (38 from community contacts and 5 from hospital contacts); 25 animals were tested from households with non-diarrhoeic children; 264 animals from the community (unselected) were investigated. None of the animals had clinical diarrhoea.

dsRNA extraction and PAGE

A small aliquot of faeces (0.5–1 g) was suspended (10% w/v) in 0.1 M sodium acetate buffer pH 5 with 1% SDS. After thorough vortexing of the specimen, a phenol-chloroform extraction of viral RNA was carried out according to Herring et al. [28], viral RNA was precipitated with ethanol and resuspended in a small volume of water. Aliquots of RNA were electrophoresed on polyacrylamide gels (10% separation gel with a 3% stacking gel) under constant current of 8 mA for 16 h in a discontinuous buffer system without SDS according to Laemmli [32] and silver-stained for detection of electropherotype pattern of rotavirus dsRNA according to Follett et al. [16].

ELISA for subgrouping

Monoclonal antibodies as ascitic fluids (AF) specific for Group A rotaviruses (AF A3M4), subgroup I virus (AF 155), and subgroup II viruses (AF 631) were provided by Dr. T. Flewett, Regional Virus Laboratory, East Birmingham Hospital, U.K. ELISA was performed using

aliquots of 10% wt/vol suspensions in PBS of each rotavirus positive specimen as described by Follett et al. [16]. Briefly, 96 well microtiter plates were coated with hyperimmune rabbit serum (1:5000 dilution) in 0.05 M carbonate bicarbonate buffer pH 9.6; test antigen was added into 6 wells for each specimen in presence of 0.1% Tween 20 and 0.005 M EDTA; each monoclonal antibody (Gr A, SGI, SGII specific) was added into duplicate wells at a dilution of 1:10000 in PBS-0.1% Tween 20–1% BSA; the substrate used was p-nitrophenyl-phosphate in diethanolamine buffer. The microtiter plates were washed six times with PBS-0.1% Tween 20 between each successive step in the ELISA procedure as appropriate. The optical density (O.D) values were read in a Titertek Multiscan Plus ELISA reader at a wavelength of 405 nm. A ratio of 2.0 or higher of the readings (SGI/SGII or SGII/SGI) was used to classify each strain as of subgroup I or II, respectively.

ds RNA RT–PCR

Selected faecal specimens were processed for extraction of double stranded RNA suitable for reverse transcription (RT) and polymerase chain reaction (PCR) for the amplification of the VP7 gene [53]. Briefly, faeces was resuspended in 2 volumes of PBS and clarified by low speed centrifugation; the supernatants were extracted with an equal volume of Arcton; the aqueous phase was next layered onto a 30% sucrose cushion, and virus was pelleted by ultracentrifugation in a Beckman SW28 swingout rotor for 2 h at 50000 **g**. The virus pellet was treated with 0.5% SDS in 0.1 M acetate buffer pH 5.0 and extracted with phenol chloroform for isolation of ds RNA. The RNA was ethanol precipitated and resuspended in a small volume of distilled water.

Reverse transcription was carried out as follows: 2 µl of ds RNA extracted from purified virus of an isolate (M48) of the 1988/89 outbreak was mixed with 1 µl of primer mix (50 ng of each primer), 3.5 µl H_2O and 2 µl of dimethylsulfoxide (DMSO), heated at 95 °C for 3 mins and snap frozen in dry ice. The 3′ half of each primer was complementary to either the 3′ end of the VP7 gene plus strand or the 3′ end of the VP7 gene minus strand of group A rotaviruses. The 5′ end part of the primers carried several restriction endonuclease sites. The VP7-specific primers were a gift from M.A. McCrae, Department of Biological Sciences, University of Warwick, with sequences as described by Xu et al. [52]. The denatured preparation was mixed with 12.5 µl of 2 × reaction buffer (100 mM Tris-HCl pH 8.3, 14 mM $MgCl_2$, 80 mM KCl, 2 mM DTT, 0.2 mg/ml gelatin, 0.4 mM each of dATP, dCTP, dGTP, and dTTP); 10 units of Avian myeloblastosis virus (AMV) reverse transcriptase (Pharmacia) diluted in 4 µl of reaction buffer was added and the reaction was incubated at 42 °C for 30 min.

Polymerase chain reaction (PCR) for amplification of gene 8 from the RT product (5 µl) of isolate M48 was carried out in a volume of 25 µl using 50ng of each Group A rotavirus VP7 gene primer in reaction buffer (10 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml gelatin, 0.2 mM each of dATP, dCTP, dGTP, dTTP) with 1 unit of Taq polymerase enzyme (Boehringer, Mannheim). The reaction mixture was overlaid with mineral oil, and 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 4 min were carried out in a Techne Model PHC II Thermal Cycler. A positive control (gene 8 *plasmid* DNA of UK strain, a gift of M. A. McCrae) and a negative control (without template) were included. PCR products (10 µl aliquots) were separated by electrophoresis on a 1.2% agarose gel beside the 1 kb DNA ladder (BRL) as molecular size marker and stained with ethidium bromide followed by visualization of the product over an UV transilluminator.

Cloning of VP7 gene of M48

The PCR product was cloned by direct ligation into PCR™ vector; (Invitrogen Corp., San Diego): the vector is supplied as a linear molecule with 3′ dT overhangs ready for

insertion of a PCR product having 3′ dA overhangs, with EcoRI sites flanking the cloning site. Briefly, the PCR product (1 μl) was added to a 10 μl ligation reaction with T4 DNA ligase and 25 ng of pCR vector, and incubated overnight at 12 °C. 1 μl of the ligation reaction was added to 50 μl of competent cells (*E. coli* INVF', Invitrogen Corp.) with 2 μl β-mercaptoethanol and transformed. An aliquot of the transformed cells was plated on 2TY-Ampicillin (50 μg/ml)-Xgal (1 mg/ml)-agar plates, to select the ampicillin-resistant recombinants after overnight incubation at 37 °C [42]. Minipreps of plasmid DNA were digested with EcoRI to select positive clones containing VP7 gene inserts of M48. The inserts were sequenced by the chain termination method of Sanger et al. [43] using ^{35}S-dATP and the sequenase version 2 kit (United States Biochemical) according to Tabor and Richardson [48]. The M48 VP7 gene clone was digested with restriction enzymes EcoRV (single digest) and Bgl II and Hind II (double digest) to get overlapping fragments which were subcloned into pBluescript KS-vector. All subclone overlapping regions were sequenced across, and the complete nucleotide sequence of the VP7 gene of M48 was obtained.

Sequence analysis

Sequence information was entered into the computer using a Graf Bar Mark II digitizer and analysed using programmes according to Staden [44] to derive the consensus sequence from several contigs. The nucleotide sequence was compared with other sequence data using the GAP programme of the Genetics Computer Group [12]. The GenBank accession no. of the VP7 gene sequence is L11605.

Results

Sequence of VP7 gene of the Manipur rotavirus strain M48

Double-stranded RNA of a long electropherotype subgroup I rotavirus isolate (M48) from the 1988/89 outbreak in Manipur was reverse-transcribed using VP7 gene-specific primers and amplified by the polymerase chain reaction (PCR) as described in methods. The PCR product was of the expected size of approximately 1.1 kbp. The complete nucleotide and deduced amino acid sequences are shown in Fig. 1.

The VP7 gene of M48 is 1062 nucleotides (nt) long with a potential open reading frame (ORF) beginning at nt 49–51 (ATG) and ending at nt 1027–1029 (TAG), coding a polypeptide of 326 amino acids (aa) in length (Fig. 1). The untranslated regions of the gene are 48 nt at the 5′ end and 33 nt at the 3′ end. A second ORF coding for a polypeptide of 297 aa in length begins at nt 136–138 and ends at nt 1027–1029. The two ORFs are in frame. Two potential glycosylation sites were found at amino acid positions 69–71 (NST) and 238–240 (NIS) as for most other G types. A third glycosylation site at position 146–148 (NTS) is specific for G2 rotaviruses [31]. Cysteine residues were conserved in eight aa positions, namely 82, 135, 165, 191, 196, 207, 244, and 249; an additional cysteine was found in aa position 289 (Fig. 1). Conserved proline residues were in positions 58, 86, 112, 131, 167, 197, 254, 275 and 279; two additional prolines were found at aa position 46 and 266 (Fig. 1).

Comparison of the VP7 gene of M48 with corresponding genes of other rotaviruses

Table 1 demonstrates that the M48 VP7 gene is very closely related to that of the G2 serotype strains RV-5 [14] and the almost identical S2 strain [27], the

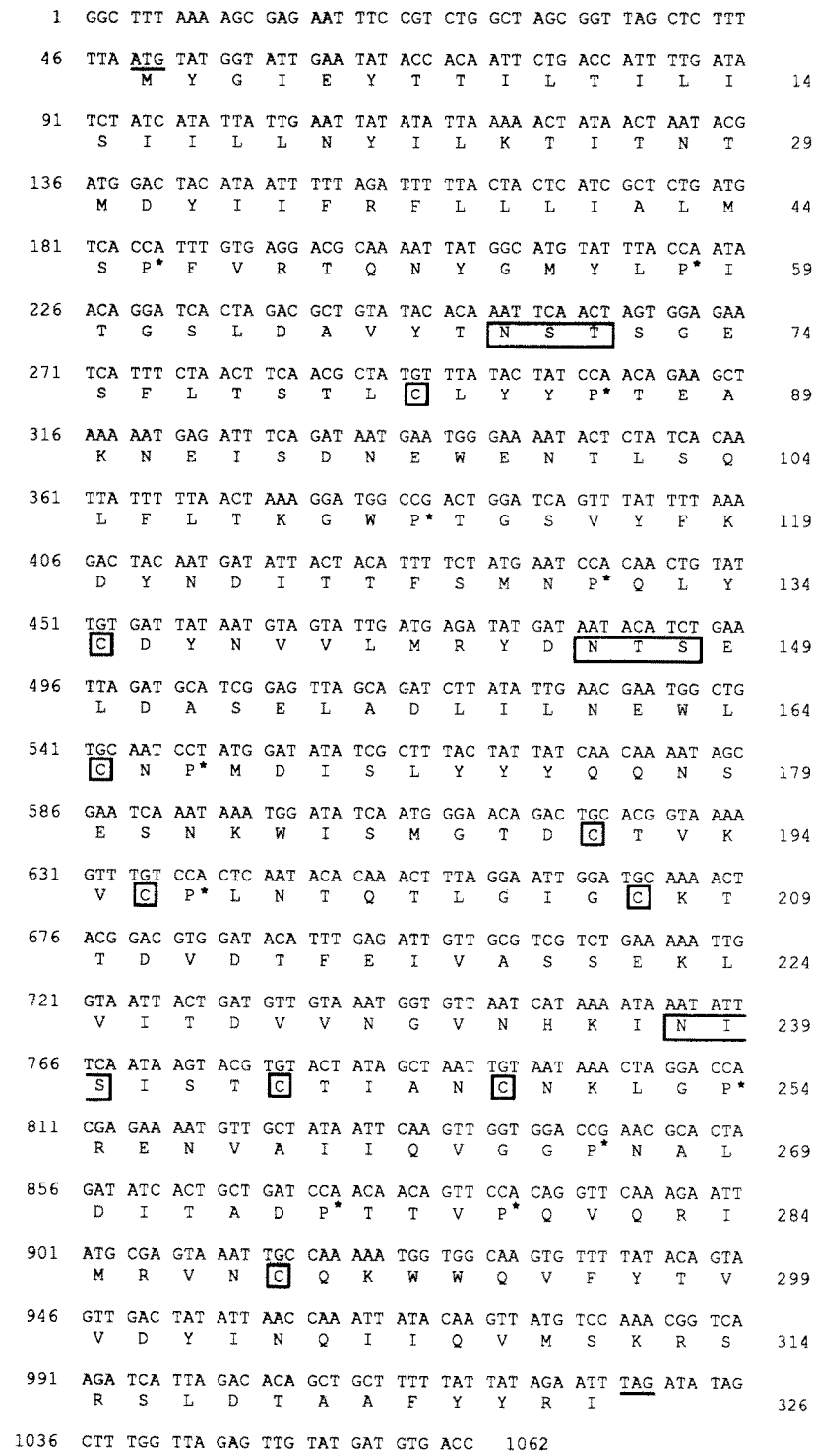

Fig. 1. Nucleotide and predicted amino acid sequences of the VP7 gene of rotavirus isolate M48. Initiation and termination codons are underlined. Potential glycosylation sites are boxed, conserved/additional cysteines are indicated in squares, and conserved/additional prolines by asterisks

Table 1. Comparison of nucleotide and predicted amino acid sequences of the VP7 gene of rotavirus strain M48 with corresponding genes of established rotavirus G types

Strain	G Serotype	% Homology with VP7 of strain M48	
		nucleotide sequence	amino acid sequence
Wa	G 1	74.5	74.6
S2	G 2	98.0	97.9
SA11	G 3	75.0	74.9
Gottfried	G 4	72.6	70.6
OSU	G 5	73.6	73.1
NCDV	G 6	72.8	74.0
Ch2	G 7	62.2	57.0
B37	G 8	73.4	74.0
WI61	G 9	75.0	76.1
B223	G10	73.7	72.8
YM	G11	75.5	74.6
L26	G12	73.8	75.5
L338	G13	73.1	71.3
F123	G14	73.4	73.7

The published sequences of the following strains were used (G types and GenBank accession data in brackets): Wa (G1; K02033), Richardson et al. [39]; S2 (G2; M11164), Gunn et al. [27]; Sa11 (G3; V01546), Both et al. [7]; Gottfried (G4: X06759), Gorziglia et al. [24]; OSU (G5; X04613), Gorziglia et al. [23]; NCDV (G6; M12394), Glass et al. [22]; Ch2 (G7, X56784), Nishikawa et al. [37]; B37 (G8; J04334), Hum et al. [29]; WI61 (G9, no accession no.), Green et al. [25]; B223 (G10; X52650), Xu et al. [53]; YM (G11; M23194), Ruiz et al. [41]; L26 (G12: M36396), Taniguchi et al. [49]; L338 (G13; D00843), Browning et al. [8]; and FI23 (G14; M61876), Browning et al. [9]

Numbers indicate percent of homology for nucleotide and predicted amino acid sequences

nucleotide sequence homology being 98.0% and the predicted amino acid homology 97.9% (for S2). This finding classifies the M48 rotavirus as a G2 type. The relationship to VP7 genes of other known serotypes was much lower (ranging from 62.2 to 75.5%). In detail, the VP7 of M48 differed by only 7 amino acids (in positions 44, 87, 96, 152, 247, 289, and 290) from that of the S2 strain (Table 2). Four of the changes were in the recognised antigenic sites A, B and D (aa positions 39–50 (A), 87–101 (B) and 143–152(D), according to Green et al. [26]; Table 2). The other 3 amino acid changes were in the carboxy terminal region of VP7. In addition, there were 15 silent nucleotide changes (Table 2).

Epidemiology of rotaviruses in Manipur 1989–1992

The incidence of rotavirus isolations during the epidemiological survey between 1989 and 1992 in field areas as well as hospitals in Manipur is shown in (Table 3). 1397 faecal specimens were screened for rotaviruses, and 288 were found positive

Table 2. Positions of differences in nucleotides and predicted amino acids between the VP7 genes of rotavirus strains M48 and S2

Nucleotide position number	change S2 → M48		Amino acid position number	change S2 → M48		antigenic region
11	A	G	UR[a]			
24	A	T	UR			
153	C	T				
171	T	C				
180	A	G	44	I	M	A
228	G	A				
249	T	C				
307	G	A	87	A	T	B
334	G	A	96	D	N	B
503	T	C	152	V	A	D
561	A	G				
582	C	T				
787	C	G				
788	G	C	247	R	A	
795	C	T				
813	G	A				
853	T	C				
915	G	C	289	W	C	
916	A	C	290	K	Q	
1043	C	T	UR			
1047	T	G	UR			
1054	A	G	UR			

The S2 sequence is from Gunn et al. [27]. The antigenic regions are according to Green et al. [26]

[a] Untranslated region

by dsRNA PAGE. The majority of the isolates had conventional combinations of electropherotype and subgroup (Table 3): 166 isolates showed long electropherotype pattern, 117 were of short electropherotype and 5 were of mixed electropherotypes. Subgroups were also tested using subgroup I (SGI), subgroup II (SG II) and (for control) group A specific monoclonal antibodies (mabs; [16]). Of the 166 isolates with long electropherotype, 6 were of SGI showing that these viruses were still prevalent in low frequency. Besides, there were 94 rotaviruses of SGII (58%), one specimen reacted with SGI and II mabs, one was only reactive with the group A mab, and 64 were non-reactive with group A, SGI or SGII mabs. Of the 117 isolates with short electropherotype, 90 were SGI (77.7%), one reacted with SGI and II mabs, one reacted with SGII, and 19 (16.2%) were non-reactive with group A, SGI or SGII mabs. 6 specimens of short electropherotype were not available for subgroup analysis. Five isolates were of mixed electropherotype patterns.

Table 3. Rotavirus isolates in Manipur, 1989–1992. Incidence in cases of diarrhoea, direct contacts and controls

Period	Cases [%]	Contacts [%]	Controls [%]	RNA Electropherotype [%] long	short	long[c]	mixed
A. Field areas							
9/89–11/90	15/111 [13.5]	1/103 [1.0]	0/228 [0]	8	8	0	0
12/90–7/91	5/54 [9.3]	0/17 [0]	3/61 [4.9]	8	0	0	0
8/91–3/92	8/42 [19.0]	0/10 [0]	0/38 [0]	1	4	1	2
Subtotal	28/207 [13.5]	1/130 [0.8]	3/327 [0.9]	17 [53.1]	12 [37.5]	1 [3.1]	2 [6.3]
B. Hospitals							
Regional Medical College, Imphal							
11/89–11/90	102/305 [33.4]	0/17 [0]	11/83 [13.3]	58 [51.3]	49 [43.4]	5 [4.4]	1 [0.9]
12/90–5/91	48/141 [34.0]	0/6 [0]	8/47 [17.0]	54 [96.4]	2 [3.6]	0	0
6/91–3/92	67/89 [75.2]	–	–	24 [35.8]	41 [61.2]	0	2 [2.9]
Subtotal	217/535 [40.5]	0/23 [0]	19/130 [14.6]	136 [57.6]	92 [38.9]	5 [2.1]	3 [1.3]
Jawaharlal Nehru Hospital, Imphal							
12/89–5/90	18/40 [45.0]	–	–	7	11	0	0
District Hospital, Bishenpur							
12/89	2/5 [40.0]	–	–	0	2	0	0
Total	265/787 [33.7]	1/153 [0.7]	22/457 [4.8]	160[a] [55.5]	117[b] [40.6]	6[c] [2.1]	5[d] [1.7]

288/1397 faecal specimens tested were positive for rotaviruses by dsRNA PAGE

The reaction of these isolates with subgroup I, subgroup II, and group A specific monoclonal antibodies, is summarised briefly: [a]94 (SGII), 1 (SGI&II), 1 (Group A only; non SGI – non SGII). 64 (non reactive to SGI, SGII or Gr A). [b]90 (SGI), 1 (SGI&II), 19 (non reactive to SGI, SGII or group A), 1 (SGII), 6 (not tested). [c]All 6 were SGI. [d]2 (SGII), 1 (SGI), 2 (non reactive towards SGI, SGII or Gr A)

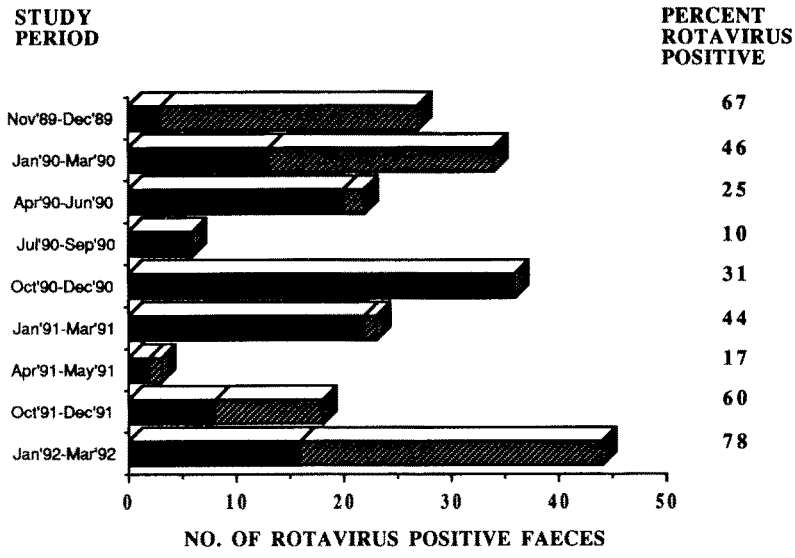

Fig. 2. Seasonal prevalence of rotavirus isolates obtained from hospitalised children with diarrhoea in Manipur, 1989–1992 (no samples were collected during June to September 1991). The electropherotypes are indicated: ■ long, ▨ short. The prevalence figures (rotavirus positive faeces as percent of faeces investigated) are indicated at the right

Only 3 out of 327 faecal specimens (= 0.9%) of nondiarrhoeic control groups of the field study isolates yielded rotavirus. By contrast, 19 out of 130 hospitalised children without diarrhoea (= 14.6%) shed rotaviruses (Table 3).

A seasonal prevalence pattern of hospitalised cases of rotavirus diarrhoea was observed during the winter months (Fig. 2). Short electropherotype viruses prevailed in 1989/90 and 1991/92 whereas long electropherotype viruses were most prominent in 1990/91. This pattern was also seen in the data from the community (results not shown). There was also a clearly elevated prevalence of rotaviruses as cause of all diarrhoeas during the winter months (Fig. 2). The epidemiological survey showed that the unusual long electropherotype subgroup I viruses which were found in relatively large numbers (in 38/53 isolates) during the 1988/89 outbreak occurred only sporadically (6 isolates) in Manipur in the years 1989–1992 (Table 3). Five were found in the hospital of the Regional Medical College between January and November 1990, and one in the field in February 1992. No rotavirus was detected in the 332 animal faecal specimens investigated so far.

Discussion

The characterization of human and animal rotavirus strains at the molecular level according to their G type (VP7) and P type (VP4) has resulted in the detection of hitherto unknown novel rotaviruses with various unusual combinations of electropherotype patterns and genotypic and antigenic properties

[1, 10, 18–21, 34]. For human strains a linkage has been observed between subgroup I and short electropherotype, and subgroup II and long electropherotype, while subgroup I specific, long electropherotype viruses were found to be mostly of animal origin [31, 34]. There is no particular restraint for the electropherotype – subgroup linkage of human strains as it was shown that different combinations of electropherotype and subgroup could be found by in vitro reassortment of human subgroup I and subgroup II viruses [17].

The detection of a large number of unusual long electropherotype, subgroup I rotaviruses during an outbreak of infantile gastroenteritis in Manipur, India, in 1988/89 [21] was preceded by isolated cases of human infection with such viruses reported from Japan [35, 36], South Africa [45] and Israel [1]. In exploring whether these novel rotaviruses in humans might have originated from animals by zoonotic transmission, it was shown that the strains from Japan and Israel were closely related to feline rotaviruses of serotype G3 [35, 36]. The analyses of long electropherotype subgroup I human rotaviruses from diarrhoea cases in the Philippines showed that these were of a new type G12 according to their VP7 gene, with VP4 of P4 type [15] which normally segregates with pathogenic human G2 viruses [30, 49].

The VP7 gene of the long electropherotype, subgroup I Manipur isolate M48 was closely related to the VP7 of serotype G2 strain of human rotaviruses (RV-5 and S2, ref 14 and 27, respectively), which are subgroup I human rotaviruses and have a *short* electropherotype. The VP4 gene of isolate M48 has been partially sequenced (Krishnan et al, in preparation) and was found to be closely related to the P4 type found in the human G2 viruses [30, 49]. Thus, the virulent strain M48 causing the Manipur outbreak has VP7 and VP4 genes characteristic of human G2 rotaviruses. It is therefore suggested that the long electropherotype pattern of strain M48 may have arisen by reassortment in vivo with another rotavirus of long electropherotype. This will be investigated further by Northern blotting followed by hybridization and by the molecular analysis of RNA 6 and RNA 11 of the M48 virus. Mixed infection in vivo (a prerequisite of reassortment) has been observed by Rodger et al [40] and later by Ahmed et al. [2]. Serotype G2 rotavirus strains of long electropherotype pattern have been isolated from piglets by Bellinzoni et al. [5], but at present there is no evidence that M48-like viruses are from an animal source.

Unusual rotaviruses of long electropherotype and subgroup I have also been detected amongst asymptomatic neonates in India over a period of 3 years [18, 47]. The VP7 gene of these viruses has been found to be closely related to that of serotype G10 rotaviruses [10, 13], and the VP4 genes were found to be of P11 type, which is closely related to that of bovine rotaviruses of serotype G10 [10, 18]. Human subgroup I rotaviruses of G9 specificity [51] were found to be closely related to animal rotaviruses in contrast to previously reported human rotaviruses of G9 type which were of subgroup II [31]. Human rotaviruses with VP7 genes of G6 or G10 specificity (G types normally found in cattle) have been reported [4, 10, 13, 18, 20, 51]. Bovine rotaviruses showing close relatedness to human rotaviruses of G1 and G8 specificity with subgroup I

characteristics have also been found [6, 33, 50]. Thus, there is increasing evidence of zoonotic transmission of rotaviruses to man.

The outbreak in Manipur during 1988/89 was followed up with an epidemiological survey for rotavirus infections in Manipur from 1989–1992. A seasonal prevalence of rotaviruses mainly during the winter months was recorded as reported by others elsewhere [3, 38, 40]. During this study unusual long electropherotype subgroup I viruses were found to be in circulation in the community only at a very low frequency, and these isolates await more detailed analysis. Animal faecal specimens screened so far were rotavirus negative, but there is possibly a sampling problem, and a search for animal rotaviruses, particularly in the large cattle population of Manipur (150 000), is ongoing.

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