

FIG. 3. Phylogenetic tree of the (A) capsid and (B) polymerase region of 32 Vietnamese SaVs and other reference SaVs. Two Vietnamese SaVs strain, HCMC86 and HCMC180, showed different genotypes when polymerase-based and capsid-based phylogenetic trees were constructed.

which indicates that they are the same strain. Nucleotide comparison showed that HCMC86 and HCMC180 had best identities (89.8–94.2%) with GII.1 strains in the polymerase region; however,

they had higher homology with two Pakistani GII.4 SaV strains (Karachi/874 and Karachi/928) than GII.1 strains (94.2% vs. 67.7–68.1%) when a capsid-based comparative analysis was performed (Table 6).

TABLE 6  
Percentage nucleotide homology of the polymerase and capsid region\* of Vietnamese SaV strains and other reference strains

|                          | I/1        |         | I/2     |         | IV      |           | II/1 |         | II/4     |        | III     |             |             |
|--------------------------|------------|---------|---------|---------|---------|-----------|------|---------|----------|--------|---------|-------------|-------------|
|                          | Manchester | HCMC286 | HCMC136 | Potsdam | HCMC143 | Hou7-1181 | Mc2  | HCMC194 | Lyon/598 | HCMC86 | HCMC180 | Karachi/874 | Karachi/928 |
| Manchester               | 99.2       | 97.4    | 80.8    | 81.5    | 65.7    | 48.9      | 49.6 | 49.6    | 49.6     | 49.6   | 48.5    | 48.5        | 45.4        |
| HCMC286                  | 97.1       | 97.4    | 81.2    | 81.9    | 66.0    | 48.9      | 49.6 | 49.6    | 49.2     | 49.2   | 48.2    | 48.2        | 45.4        |
| HCMC136                  | 97.1       | 80.4    | 80.4    | 80.4    | 65.3    | 48.9      | 49.6 | 49.6    | 48.5     | 48.5   | 48.2    | 48.2        | 45.4        |
| Potsdam                  | 84.0       | 81.1    | 81.1    | 97.1    | 63.1    | 46.0      | 45.3 | 46.0    | 44.3     | 44.3   | 42.9    | 42.9        | 41.4        |
| HCMC143                  | 84.0       | 81.1    | 100     | 63.8    | 63.8    | 47.1      | 46.4 | 47.1    | 46.0     | 46.0   | 44.6    | 44.6        | 40.7        |
| Hou7-1181                | 73.9       | 72.4    | 73.9    | 73.9    | 63.8    | 46.7      | 47.1 | 47.5    | 49.2     | 49.2   | 47.1    | 47.1        | 40.8        |
| Mc2                      | 72.4       | 69.5    | 76.8    | 76.8    | 85.5    | 85.5      | 98.9 | 98.2    | 68.1     | 68.1   | 67.3    | 67.3        | 43.5        |
| HCMC194                  | 68.1       | 65.2    | 72.4    | 72.4    | 81.1    | 95.6      | 95.6 | 97.8    | 68.1     | 68.1   | 67.3    | 67.3        | 43.2        |
| Lyon/598                 | 72.4       | 69.5    | 76.8    | 76.8    | 85.5    | 100       | 95.6 | 97.8    | 68.1     | 68.1   | 67.3    | 67.3        | 43.2        |
| HCMC86                   | 71.0       | 71.0    | 75.3    | 75.3    | 84.0    | 84.0      | 89.8 | 94.2    | 67.7     | 67.7   | 67.0    | 67.0        | 44.6        |
| HCMC180                  | 71.0       | 71.0    | 75.3    | 75.3    | 84.0    | 84.0      | 89.8 | 94.2    | 100      | 100    | 94.2    | 94.2        | 45.7        |
| Karachi/874 <sup>b</sup> | N/A        | N/A     | N/A     | N/A     | N/A     | N/A       | N/A  | N/A     | N/A      | N/A    | N/A     | N/A         | 45.0        |
| Karachi/928 <sup>b</sup> | N/A        | N/A     | N/A     | N/A     | N/A     | N/A       | N/A  | N/A     | N/A      | N/A    | N/A     | N/A         | 45.0        |
| PEC                      | 50.7       | 50.7    | 55.0    | 55.0    | 50.7    | 52.1      | 49.2 | 52.1    | 52.1     | 52.1   | N/A     | N/A         | 45.0        |

Best identities results of HCMC86 and HCMC180 are in shaded.

\*Nucleotide homology of polymerase region is shown in the lower left, and capsid region is in the upper right. Genogroups and genotypes are also indicated.  
<sup>b</sup>The nucleotide sequences of the polymerase region of Karachi/874 and Karachi/928 are not available.

The phylogenetic analysis also indicated clearly that HCM86 and HCM180 clustered into two different genotypes when polymerase-based and capsid-based nucleotide phylogenetic trees were constructed. Altogether, these two Vietnamese SaV strains are GII.1/GII.4 recombinant strains.

### Discussion

In this study, we reported the detection of NoVs and SaVs among diarrheic children in the Children's Hospital 1, Ho Chi Minh City, during 2005–2006. With the overall detection rate of 6.4% and 1.2%, respectively, NoVs and SaVs continued to be viral agents causing acute gastroenteritis in children in the southern part of Vietnam. Although these detection rates were slightly lower than those of the studies in developed countries [37, 38], the results in this study were similar to those of the epidemiological studies conducted previously at the same hospital [29, 30], and also comparative with other surveillances in other developing countries [39, 40]. Despite difference of time, the detection of NoVs and SaVs with similar proportions in the southern part of Vietnam indicated that these viruses have circulated stably in the area. NoV GI was not found in this study, and this result was in agreement with the previous study [29]. The absence of NoV GI in epidemiological surveillance was also reported elsewhere [18, 34]. The primer sets using in this study have been used to screen caliciviruses in other surveys, and they successfully identified NoV GI in the studied samples. Therefore, the inability to detect NoV GI strains in this study might have resulted from the absence of this virus within the collected fecal specimens.

In temperate climate countries, NoVs are usually identified in the winter time [36, 38], whereas in tropical countries, the seasonal pattern of NoVs is not clear. In this survey, NoVs was found all year round, except in April and July. Moreover, 62.5% of NoVs were detected from May to October, indicated that this virus prevailed during the rainy season. This result was concordant with that of the previous study during 2002–2003 [29], and slightly different from the result of the 1999–2000 survey, in which, NoVs prevailed at the end of the rainy season and the first half of the dry season [30]. However, the results of the 1999–2000 survey based on the specimens that were negative for other common viral agents, therefore, the absence of NoVs strains, if any, which were mixed infection with other viruses, might make the feature of monthly distribution of NoVs incomplete.

NoV GII.4 was the most common (50.0%) genotype among NoV strains detected in this study. Previous studies in Ho Chi Minh City also found NoV GII.4 in 78% and 82.1% of samples [29, 30], confirming the predominance of this genotype.

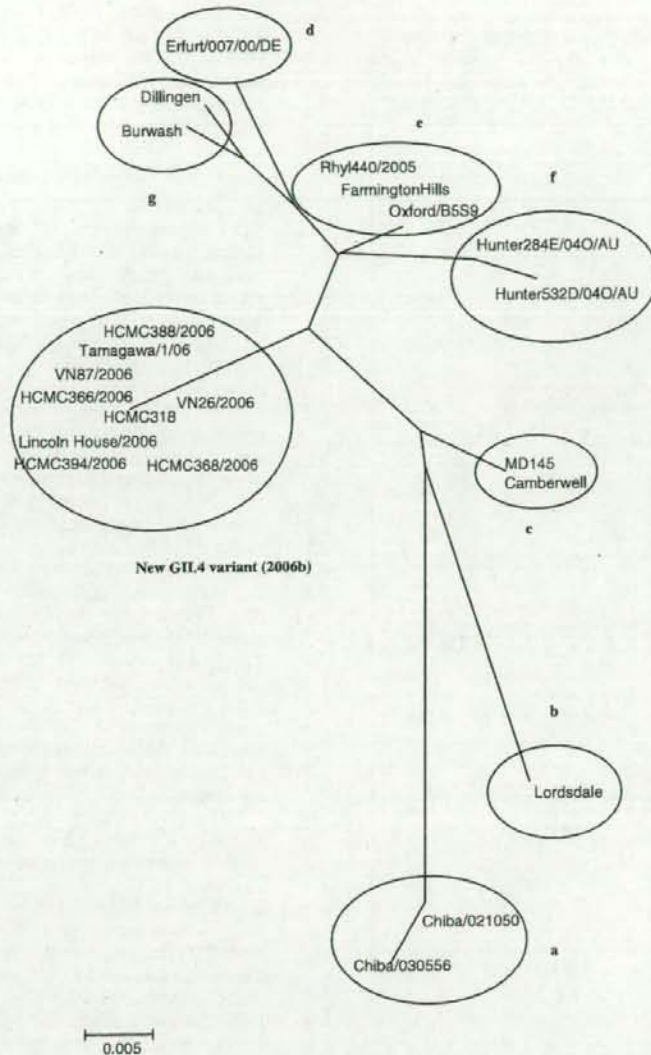


FIG. 4. Unrooted phylogenetic tree of new NoV GII.4 variant identified in this study and other GII.4 lineages. Vietnamese NoVs detected from another study [41], VN26 and VN87, as well as other reference strains, Tamagawa/1/06 and Lincoln House/2006, are included. The classification is based on Phan *et al.* [14].

However, 7 out of 16 Vietnamese GII.4 strains in this study belonged to a distinct cluster which has been determined as a novel GII.4 variant, 2006b [41] (Figs 1 and 4). These strains were firstly identified in September, and continued to be detected until the end of the surveillance, suggesting that these

viruses have been continuing to prevail in this area in the coming year. Different genotypes of NoVs and SaVs were determined in this study, and among them, several genotypes have not been reported formerly in Vietnam (NoV GII.6, SaV GI.2 and GII.4). Of interest, SaV GII.4 was only



reported in two unique Pakistani strains collected in 1992 and 1994, respectively [42]. On the other hand, the 'new variant' designated GII.b NoVs, which has been detected in Europe in the beginning of 2000s and then identified in Asia [36, 38, 43, 44], could not be found in this study. A larger number of specimens, as well as an attempt to collect fecal samples from different places in Vietnam is needed for confirming the absence of this virus in the country.

Although detected in several epidemiological studies, and being considered as important viral agent causing acute gastroenteritis in young infants and children, this was the first time, to our knowledge that the clinical manifestations of NoV infections were described in Vietnamese pediatric patients. The clinical features of NoV-associated acute gastroenteritis observed among patients in this study were similar to those of other reports, including diarrhea with watery stool, vomiting and fever [18, 20]. Although the results of this study were comparable to another study conducted in Japan [18], the mean duration of diarrhea and maximum episodes of diarrhea per day in Vietnamese children were observationally higher than those of Finnish children (4.4 days vs. 2 days, 6.5 times/day vs. 4 times/day) [45]. The difference might be explained by the population studied. In this study, we collected samples from patients who sought to the hospital, whereas the survey carried out in Finland was a community-based study. Therefore, although both were classified as moderately severe diseases (8–10 points) [45], the mean severity score in Vietnamese patients was obviously higher than that of Finnish children (9.8 vs. 8).

A comparative analysis was performed in order to see the difference in severity among several groups of patients, however, only the mean scores were statistically different between inpatients and outpatients. This situation was also observed among astrovirus positive patients described previously [31].

The clinical manifestation of SaV infection in this study could not be demonstrated because only one medical record among two SaV monoinfection cases was available. This patient suffered from an 8-day diarrhea, with maximum episode of diarrhea was 20 times per day and high fever up to 39°C. This feature was much different from other reports, which described SaV-associated diarrhea to be a mild disease. More clinical data on larger number of patients are needed in order to identify properly the clinical features of SaV infection in Vietnamese children.

RNA recombination plays a key role in virus evolution and it shapes a good deal of the virus diversity [46]. Recombinant NoV strains were increasingly found in epidemiological surveillances throughout the world [22, 23, 38, 43], including Vietnam [30]. In this survey, various types of

recombination in NoVs were identified. Of interest, the GII.6b (polymerase)/GII.6c (capsid) recombination was first reported in this study. Similarly, the recombinant GII.1/GII.4 SaV strain detected in this survey has not been described elsewhere. Half of NoV strains, and one out of six SaV strains were identified as recombinant viruses, thus indicates that recombination is not a rare event, and the caliciviruses circulating in Vietnam have a trend to be more diverse.

The results of this study highlight the impact of caliciviruses in diarrheal diseases among children in Ho Chi Minh City, and are the first to describe the clinical manifestations of NoV infections in Vietnamese children. The data of nucleotide analysis from this study could provide useful information for knowledge on caliciviruses characteristics.

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## Risk for HIV-1 infection is not associated with Repeat-Region polymorphism in the DC-SIGN neck domain and Novel Genetic DC-SIGN Variants among North Indians

Anurag Rathore<sup>a</sup>, Animesh Chatterjee<sup>a</sup>, Vikas Sood<sup>b</sup>, Sohrab Z. Khan<sup>b</sup>, Akhil C. Banerjee<sup>b</sup>, Naohiko Yamamoto<sup>c</sup>, Tapan N. Dhole<sup>a,\*</sup>

<sup>a</sup> Department of Microbiology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Raebareilly Road, Lucknow, India

<sup>b</sup> Laboratory of Virology, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi, India

<sup>c</sup> Department of International Health, Nagoya University School of Medicine, Japan

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### Abstract

**Background:** Several genetic factors have been related to HIV-1 resistance, the homozygosity for a mutation in CCR5 gene (CCR5Δ32 allele) is presently considered the most relevant one. The C-type lectin, DC-SIGN efficiently binds and transmits HIV-1 to susceptible cell *in trans* thereby augmenting the infection. A potential association of the DC-SIGN neck domain repeats polymorphism and risk of HIV-1 infection is currently under debate.

**Methods:** Genetic risk association study was conducted in HIV-1 exposed seronegative (HES;  $n=50$ ) individuals, HIV-1 seronegative (HSN;  $n=314$ ) healthy control and HIV-1 infected seropositive patients (HSP;  $n=190$ ) for polymorphism in neck domain of DC-SIGN gene. The DC-SIGN genotypes were identified by PCR from DNA extracted from peripheral blood and confirmed by sequencing. Fisher exact or  $\chi^2$  test was used for statistical analysis.

**Results:** One HSN and HSP individual who were heterozygous (7/8) with respect to DC-SIGN repeat regions were found. The DC-SIGN neck repeat polymorphism among North Indian individuals was not associated with susceptibility to HIV-1 infection. Furthermore, inheritance study of heterozygous mutation (7/8) in HSN individual's family showed that one parent, two brothers, one sister and one daughter were heterozygous (7/8) for DC-SIGN mutant allele. Sequence analyses of DC-SIGN exon 4 repeat region of randomly selected 25 North Indian individuals from HSP, HSN and HES revealed four conserved intronic mutations. These mutations were at nucleotide position 1283, 1306, 1308 upstream and 1906 downstream of the DC-SIGN exon 4 repeat region when compared with the wild type sequence (NCBI Acc. No. AF209479).

**Conclusion:** The polymorphism in DC-SIGN neck repeats region was rare and not associated with HIV-1 susceptibility among North Indians. Sequencing analysis of DC-SIGN gene confirmed four novel genetic variants in intronic region flanking exon 4 coding region.

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**Keywords:** DC-SIGN; Innate immunity; HIV-1 infection; Intron; Exon; DC-SIGN gene

### 1. Introduction

Genetic polymorphism in human genes can influence the risk for HIV-1 infection and disease progression [1,2]. There are some individuals who remain seronegative despite high risk and/

or multiple exposures to HIV-1 [3,4]. Although several factors have been related to HIV-1 infection resistance, the possible genetic mechanism underlying this resistance is the homozygous presence of a 32 bp deletion in CCR5 gene (CCR5 Δ32), i.e. the main co-receptor used by the macrophage (M)-tropic strain of the virus to infect peripheral blood mononuclear cells [5].

The dendritic cell receptor, DC-SIGN (dendritic cell-specific ICAM-3 grabbing non-integrin, encoded by CD209) is a type II membrane associated C-type lectin that binds HIV-1 envelope glycoprotein gp120 in a CD4-independent manner and function

\* Corresponding author. Department of Microbiology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Raebareilly Road, Lucknow-226014, India. Tel./fax: +91 522 2668100.

E-mail address: [tapedhole@yahoo.co.in](mailto:tapedhole@yahoo.co.in) (T.N. Dhole).



as a Trojan horse to enhance trans-virus infection to host cells [6]. DC-SIGN is of particular interest because it recognizes a plethora of pathogens such as *Mycobacterium tuberculosis*; *Helicobacter pylori*, *Klebsiella pneumoniae*, HIV-1, Ebola virus, Cytomegalovirus, Hepatitis C virus, Dengue virus, SARS-coronavirus and parasites like *Leishmania pifanoi* and *Schistosoma mansoni* [7]. Cotransfection of DC-SIGN with HIV-1 demonstrated 95–99.5% inhibition of viral production from host cells and can also effectively inhibit 90–95% of HIV-1 generation from infected cell [8]. DC-SIGN is organized into 3 domains: an N-terminal cytoplasmic region, a neck region containing 7 tandem repeats of the 23 amino acid sequence, and a C-terminal domain with homology to C-type lectins [9]. The neck region of DC-SIGN may be important in determining the orientation of the carbohydrate-binding C-type lectin domain and may therefore have an impact on ligand specificity [10]. Polymorphism analysis of the numbers of repeats present in this neck region suggested that tandem repeats in the neck region are variable and predominantly consisted of 7 repeats among the Caucasians [11]. However, novel variations in the DC-SIGN repeat region were identified which were rare. Cohort studies of HIV-1 seronegative, HIV-1 seropositive and repeatedly exposed seronegative individuals suggest that heterozygosity in the DC-SIGN repeat region may have protective effect on transmission of HIV-1 [12] whereas no associations could be established in recent studies on Thais individuals [13].

Since, the impact of DC-SIGN neck repeat polymorphism on HIV-1 disease susceptibility/transmission have yielded conflicting results, we investigated the effect of variable number of tandem repeat (VNTR) polymorphism in DC-SIGN neck region on HIV-1 susceptibility among North Indian individuals.

## 2. Materials and methods

### 2.1. Patient selection

All the subjects with similar ethnicity were randomly selected from same source population. Demographic profiles of the study groups are given in Table 1. 190 HIV-1 seropositive patients (HSP) were enrolled from the outpatients attending the clinics of Sanjay Gandhi Post Graduate Institute of Medical Sciences, India from Jan. 2004 to Nov. 2006. Most of the patients were from the state of Uttar Pradesh in North India. 320 age-matched controls were healthy staff members of institute with HIV-1 seronegative status. 50 HIV-1 seronegative individuals with history of repeated sexual intercourse (twice a week) without any protection with HIV-1 infected partners for at least 1 y were recruited in HIV-1 exposed seronegative group (HES). HIV-1 seronegative status of the HES subjects was confirmed by Western blot at the regular interval of three months till one year. After an informed consent, 5-ml blood sample was

Table 1  
Demographic profile of the study groups

|                      | HSP         | HSN         | HES        |
|----------------------|-------------|-------------|------------|
| No. of individuals   | 190         | 320         | 50         |
| Male no. (%)         | 169 (88.94) | 275 (85.93) | 44 (88)    |
| Female no. (%)       | 21 (11.05)  | 45 (14.06)  | 6 (12)     |
| Median age (range)   | 58 (42–74)  | 56 (38–60)  | 59 (35–55) |
| Heterosexual no. (%) | 190 (100%)  | N.D.        | 50 (100%)  |
| Homosexual no. (%)   | 0 (0%)      | N.D.        | 0 (0%)     |

HSP = HIV-1 Seropositive, HSN = HIV-1 Seronegative, HES = HIV-1 Exposed Seronegative, N.D. = Not Detected.

Table 2

Distribution of CCR5 delta 32 genotypes/allele among HIV-1 Seropositive, HIV-1 Seronegative and HIV-1 Exposed Seronegative individuals

| CCR5 delta 32             | HSP (n=190) | HSN (n=320) | HES (n=50) |
|---------------------------|-------------|-------------|------------|
| wt allele (%)             | 380 (100)   | 634 (99.06) | 100 (100)  |
| delta 32 allele (%)       | 0 (0)       | 6 (0.93)    | 0 (0)      |
| CCR5 wt/wt (%)            | 190 (100)   | 314 (98.12) | 50 (100)   |
| CCR5 delta32/wt (%)       | 0 (0)       | 6 (1.87)    | 0 (0)      |
| CCR5 delta 32/delta32 (%) | 0 (0)       | 0 (0)       | 0 (0)      |

Percentage values given within parenthesis.

HSP: HIV-1 Seropositive; HSN: HIV-1 Seronegative; HES: HIV-1 Exposed Seronegative.

taken in EDTA for serological and polymorphism studies. Necessary institutional biosafety and ethical clearances were obtained.

### 2.2. Determination of HIV-1 status

Altogether 560 individuals were screened for their HIV-1 status by primarily screening with ELISA (Vironostika, HIV Uni-FormII Ag/Ab, Biomérieux, Netherlands) and subsequently confirmed with Western Blot (LAV Blot I, BioRad, France).

### 2.3. Genomic DNA isolation

The genomic DNA samples were obtained from 0.2 to 0.3 ml peripheral whole blood by using QIAamp Blood kit (Qiagen, San Diego CA) according to the protocols supplied by the manufacturer. Usually ~0.1 µg genomic DNA was used for the PCR.

### 2.4. Genotyping of CCR5 delta 32 mutation

CCR5 delta 32 genotype was determined by sizing amplicons that include the entire region of the deletion as described previously [14]. Polymerase Chain Reaction (PCR) was conducted in a 25 µl reaction containing 50 ng of genomic DNA, 5 pmol of each primer, 175 µM dNTPs, 1.5 mM MgCl<sub>2</sub>, 10X PCR buffer and 0.5 U of Taq polymerase (Roche). Thermocycling procedure (PerkinElmer 9600) consisted of initial denaturation at 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 52 °C for 45 s and 72 °C for 1 min and final extension of 72 °C for 7 min. Amplicons were visualized by ultraviolet transillumination in 2% agarose gel containing ethidium bromide. The sense primer was 5'-TGTTTGGCGTCTCCAG-3' and antisense was 5'-CAC AGC CCT GTG CCT CTT-3', which result in a 233 bp product for the wild type amplicon and 201 bp for the deletion product.

### 2.5. Genotyping of DC-SIGN neck repeat

The DC-SIGN neck repeat region was amplified from genomic DNA as described previously [12]. PCR amplification was performed using forward primer: 5'-CCA CTT TAG GGC AGG AC-3' and reverse primer: 5'-AGC AAA CTC ACA CCA CAC AA-3' in a volume of 25 µl containing 0.00025 µmol/µl dNTPs, 1.0 µmol/µl each primer, 1 µl Glycerol, 0.0025 µmol/µl MgCl<sub>2</sub> and 1.0 U Taq polymerase (Roche) in a 1X reaction buffer. The cycle conditions were 5 min at 94 °C, followed by 30 cycles of 15 s at 95 °C, 7 s at 61 °C, 30 s at 72 °C and then a single cycle of 7 min at 72 °C. Wild type alleles (7/7) yielded 852 bp PCR products while heterozygous individuals (8/7) was identified by the appearance of 2 closely spaced PCR products (921 bp and 852 bp) when analyzed on a 3% agarose gel by ethidium bromide staining.

### 2.6. Cloning and sequencing of wild and mutant alleles DNA fragments

PCR generated DNA fragments of wild and heterozygous mutants were excised from the gel and were cloned directly in a T-tailed vector (pGEM T-Easy, Promega Biotech, Madison, WI). Positive clones were confirmed by EcoRI digestion and sequenced on ABI Bioprism.









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## Sequence Analysis of the Capsid Gene of Aichi Viruses Detected From Japan, Bangladesh, Thailand, and Vietnam

Ngan Thi Kim Pham,<sup>1</sup> Quang Duy Trinh,<sup>1</sup> Pattara Khamrin,<sup>1</sup> Tuan Anh Nguyen,<sup>1</sup> Shuvra Kanti Dey,<sup>1</sup> Tung Gia Phan,<sup>1</sup> Le Phuc Hoang,<sup>2</sup> Niwat Maneekarn,<sup>3</sup> Shoko Okitsu,<sup>4</sup> Masashi Mizuguchi,<sup>1</sup> and Hiroshi Ushijima<sup>1,4\*</sup>

<sup>1</sup>Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

<sup>2</sup>Department of Gastroenterology, Children's Hospital 1, Ho Chi Minh City, Vietnam

<sup>3</sup>Faculty of Medicine, Department of Microbiology, Chiang Mai University, Chiang Mai, Thailand

<sup>4</sup>Aino Health Research Center, Aino University, Tokyo, Japan

Sequence analysis of the capsid gene of Aichi viruses was performed on 12 strains detected in Japan, Bangladesh, Thailand, and Vietnam during 2002–2005. The phylogenetic tree constructed from 17 nucleotide sequences of the capsid gene of the strains studied and reference strains demonstrated that Aichi virus strains clustered into two branches. A classification of Aichi viruses based on the capsid gene was proposed, in which lineage I consists of the Aichi virus strains detected from Japan, Thailand, Vietnam, and Germany, and lineage II includes Bangladeshi strains and a Brazilian strain. *J. Med. Virol.* 80:1222–1227, 2008. © 2008 Wiley-Liss, Inc.

**KEY WORDS:** sequence analysis; Aichi viruses; capsid gene

### INTRODUCTION

Acute gastroenteritis is a common cause of morbidity and mortality worldwide. Rotaviruses, adenoviruses, astroviruses, and caliciviruses have been established as the most important etiologic agents [Glass et al., 2001]. However, there remains a "diagnostic gap," which has been attributed to less explored viral pathogens. It was suggested that unknown agents account for approximately 81% of foodborne illnesses and hospitalizations and 64% of deaths in United States [Mead et al., 1999]. Identifying new causes of enteric illness and defining the public health importance of known agents would improve foodborne disease prevention. Among the less explored viral pathogens, Aichi virus was first recognized in 1989 as the likely cause of oyster-associated gastroenteritis in Japanese patients. It is a small round virus with about 30 nm in diameter [Yamashita et al., 1991]. The virus was classified into a new genus named Kobuvirus of the family

Picornaviridae which contains nine genera: Aphthovirus, Cardiovirus, Enterovirus, Erbovirus, Hepatovirus, Kobuvirus (which includes Aichi virus and bovine kobuvirus), Parechovirus, Rhinovirus, and Teschovirus according to the International Committee on Taxonomy of Viruses [Yamashita et al., 1998; Pringle, 1999].

By using a neutralization test, the prevalence rate for antibody against Aichi strain in healthy adults and children in Aichi Prefecture, Japan was estimated and found to be 7.2% for infants and children aged 7 months to 4 years and increased with age to about 80% in persons 35 years old [Yamashita et al., 1993]. Aichi virus was then isolated in Vero cells from 6 (12.3%) of 47 patients in 5 gastroenteritis outbreaks in Japan. In addition, 5 (0.7%) of 722 Japanese travelers returning from tours to Southeast Asian countries and complaining of gastrointestinal symptoms at the quarantine station of Nagoya International Airport in Japan, and 5 (2.3%) of 222 Pakistani children with gastroenteritis, were found to be positive for Aichi virus [Yamashita et al., 1995].

The complete Aichi virus genome was determined in 1998 and proved to be a single-stranded positive-sense RNA molecule with 8,251 bases excluding a poly (A) tail; it contains a large open reading frame (ORF) with 7,302 nucleotides that encodes a potential polyprotein

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\*Correspondence to: Prof. Hiroshi Ushijima, MD, PhD, Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo Bunkyo-ku, Tokyo 113-0033, Japan.  
E-mail: ushijima-hiroshi@icm.home.ne.jp

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precursor of 2,433 amino acids [Yamashita et al., 1998]. In the year 2000, a reverse transcription-polymerase chain reaction (RT-PCR) method for the detection of Aichi virus was developed and the genetic analysis was performed using the 519 base RNA sequences at the putative junction between the C terminus of 3C and the N terminus of 3D. As a result, Aichi virus isolates have been divided into two groups: group 1 (genotype A) and group 2 (genotype B) [Yamashita et al., 2000]. Since then, basic researches into Aichi virus were consecutively performed [Sasaki et al., 2001, 2003; Yamashita et al., 2001; Nagashima et al., 2003, 2005]. Recently, Aichi virus was also detected in Brazil, Germany, Thailand, Bangladesh, and Vietnam [Oh et al., 2006; Pham et al., 2007].

It is well established that highly specific serological tests used for the diagnosis of viral infections could be developed on the basis of heterologously expressed viral structural proteins. In previous studies, however, the detection and classification of Aichi virus were based on the 3CD region coding for the non-structural protein [Yamashita et al., 2000; Oh et al., 2006; Pham et al., 2007]. Therefore, in order to research further into Aichi virus genome, this study was performed to characterize the capsid region coding for structural proteins which express the antigenicity of the virus.

## MATERIALS AND METHODS

### Clinical Specimens

Twelve fecal samples which have already known to be infected with Aichi virus determined by PCR using the Aichi virus-specific primers [Yamashita et al., 2000; Pham et al., 2007] were examined in this study. Those samples were collected from epidemiological studies/surveillances during the period of 2002–2005 in the following countries: Japan (six samples collected from July 2002 to June 2003), Bangladesh (three samples, October 2004 to September 2005), Thailand (one sample, March 2002 to December 2004), and Vietnam (two samples, October 2002 to September 2003) [Phan et al., 2005; Dey et al., 2007; Khamrin et al., 2007; Nguyen et al., 2007].

### RNA Extraction, RT-PCR, and Primer Designation

RNA extraction was first done using the QIAamp viral RNA Mini kit (QIAGEN, Inc., GmbH Hilden, Germany). The RT-PCR was then performed. To obtain the full length of 2,616 bases of the capsid gene, 5 segments were constituted and 10 primers were newly developed based on the 5 complete genomes of Aichi viruses available from GenBank and the sequence data obtained during the capsid gene characterization of Aichi virus strains detected from the four countries. Five forward primers Cap F, Cap2, Cap3, Cap4, and CapE were used in combination with reverse primers Cap1R, Cap2R1, Cap3R, Cap4R, and CapER, respectively (Table I).

### Sequence and Phylogenetic Analysis

The PCR amplicons were purified and sequenced in both directions using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer-Applied Biosystems, Inc., Foster City, CA). The primers designed for PCR were used as sequencing primers. The sequence data were collected by an ABI Prism 310 Genetic Analyzer (Perkin Elmer-Applied Biosystems, Inc.).

The comparison analysis was conducted between the obtained Aichi virus strains and other Aichi virus reference strains from Japan, Germany, and Brazil available in GenBank database. The sequence data and the phylogenesis were analyzed using BioEdit v7.0.5. A parsimony analysis was also conducted using MEGA version 3.1 to determine the evolutionary relationship among studied sequences [Kumar et al., 2004]. The method was performed using close-neighbor interchange with a random option and with 1,000 bootstrap repetitions.

The nucleotide sequences of the Aichi virus strains described in this study have been deposited in GenBank under accession numbers: EU143271 to EU143279, EU143282, EU143286, and EU143287. Reference Aichi virus strains and their accession numbers are as follows: AB010145, AB040749, AY747174, NC001918, and DQ028632.

TABLE I. Oligonucleotide Sequences of the Five Primer Pairs Used in This Study

| Primer | Sequence 5'–3'                | Sense | Position* |
|--------|-------------------------------|-------|-----------|
| Cap F  | CAG GTG CCT ACC AAG CAA AGA C | +     | 1104–1125 |
| Cap1R  | GGT GAA CTC CTG GGA CCA G     | –     | 1786–1768 |
| Cap2   | CCT CGC CTA CCC CAC CGC C     | +     | 1666–1684 |
| Cap2R1 | GAG ACC GTG GAA RGA GGA GTC   | –     | 2317–2297 |
| Cap3   | CAT AGA GGT CCC YTA YAT CTC   | +     | 2149–2169 |
| Cap3R  | CAT ACK GTG TAT GTT CCG CGC   | –     | 2760–2740 |
| Cap4   | CAG TGG CGY GGT GRA CTC G     | +     | 2618–2636 |
| Cap4R  | GCG ATG TAY GTG AAG CAC G     | –     | 3303–3285 |
| Cap E  | CTA GTC GGA CCC CAC ACC GC    | +     | 2897–2915 |
| CapER  | GGA TGG CCC AGT GGA CGT AG    | –     | 3854–3835 |

R: A or G.

Y: C or T.

K: T or G.

\*Based on the Japanese reference strain with the accession number of AB010145.



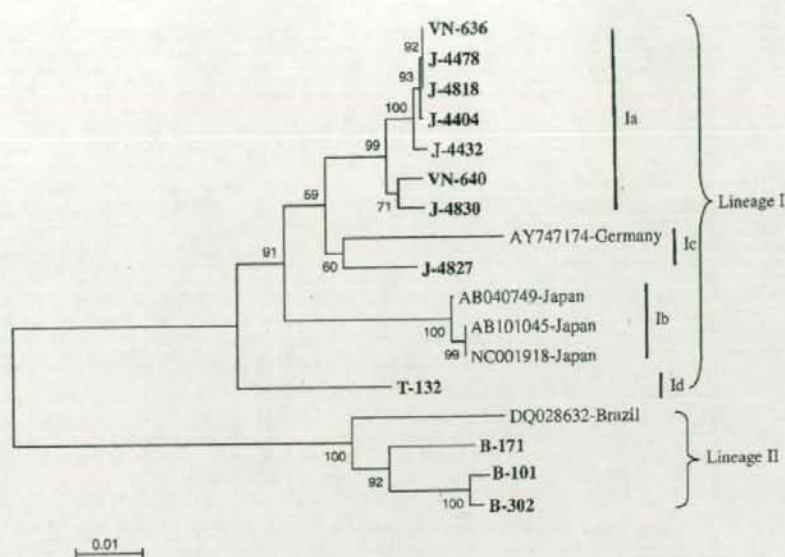


Fig. 1. Phylogenetic tree constructed from the full length sequences of the capsid gene with 1,000 bootstrap repetitions. The strains in this study are in boldface type. Abbreviations for locations: J, Japan; B, Bangladesh; VN, Vietnam; T, Thailand.

## RESULTS

Twelve full lengths of Aichi virus capsid gene sequences from the four countries including Japan (6 strains), Bangladesh (3 strains), Thailand (1 strain), and Vietnam (2 strains) were obtained for comparison with global Aichi virus strains determined previously. The full length of the capsid gene of 9 strains from Japan, Thailand, and Vietnam was 2,616 bases, while that of 3 Bangladeshi sequences contained 2,619 nucleotides.

The phylogenetic tree in Figure 1 clearly showed that all of Aichi virus strains were clustered into two major branches. One branch consisted of four sub-branches containing the studied strains from Japan, Thailand,

Vietnam and reference strains from Japan, and Germany. The remaining branch comprised of the Bangladeshi strains and one reference strain from Brazil.

Distance matrix analysis of nucleotide sequences of the capsid gene incorporated with their phylogenetic tree demonstrated two major lineages which could be designated as I and II (Fig. 1 and Table II). The relationship among lineages is clearly shown in Table II. The same lineages were defined by 3.1% and 3.3% nucleotide distances, while distances between lineages were equal to or higher than 12.7%. In addition, the mean nucleotide distances among all Aichi virus strains of three structural domains of VP0, VP3, and VP1 of the capsid gene were calculated separately and found to be 12.1%, 13.3%, and 15.2%, respectively (data not shown).

A comparison of the deduced amino acid sequences of the whole capsid gene of all Aichi virus strains showed that there were at least 14 amino acid substitutions which were lineage-specific. There was a different triple of amino acids at positions 220–222 (TSS (Threonine-Serine-Serine) located within the VP0 region of lineage I and STN (Serine-Threonine-Asparagine) in case of lineage II, and an insertion of Proline or Serine at position 224 of lineage II sequences which could not be found within the lineage I sequences (Fig. 2).

## DISCUSSION

Aichi virus virions contain three (VP0, VP3, and VP1), not four as other picornaviruses (VP1, VP2, VP3, and VP4), capsid proteins of 42, 30, and 22 kDa. No protein band corresponding to VP4 (usually 7–8 kDa) was

TABLE II. Percentages of Nucleotide Distances Among Aichi Lineages and Sub-Lineages

| Aichi virus lineage | Mean% nucleotide distance |             |             |             |             |
|---------------------|---------------------------|-------------|-------------|-------------|-------------|
|                     | Lineage I                 |             |             |             | Lineage II  |
|                     | Ia                        | Ib          | Ic          | Id          |             |
| Lineage I           | (3.3)                     |             |             |             | <b>13.3</b> |
| Ia                  | 0.6                       |             |             |             |             |
| Ib                  | 4.5                       | 0.2         |             |             |             |
| Ic                  | 4.3                       | 5.8         | 3.4         |             |             |
| Id                  | 4.8                       | 6.6         | 5.1         | NA          |             |
| Lineage II          | <b>13.4</b>               | <b>13.5</b> | <b>13.4</b> | <b>12.7</b> | (3.1)       |

Nucleotide distances within lineage I and lineage II are presented in parentheses, while distances between lineage II and every sub-lineage of lineage I are in boldface type. NA, not applicable.

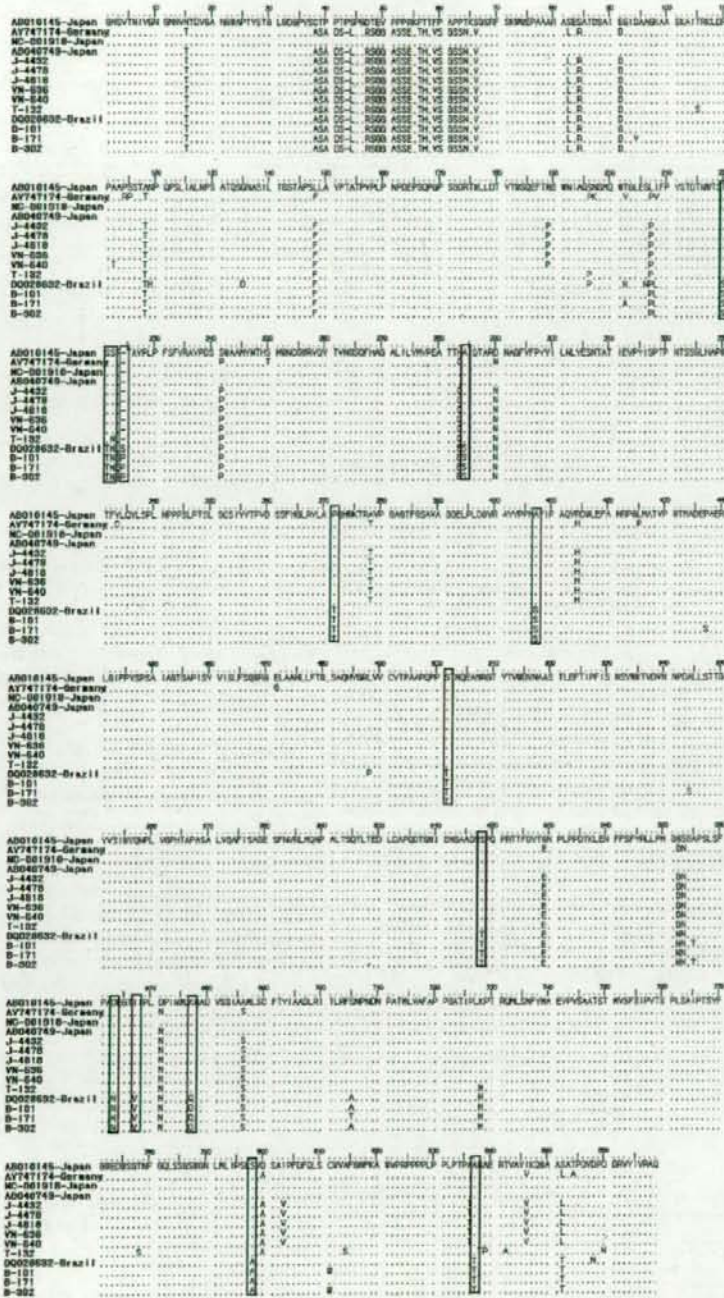


Fig. 2. Alignment of the deduced amino acid sequences of the capsid gene between the studied strains and the reference strains. Different amino acids between the two major lineages are in boxes. Abbreviations for locations: J, Japan; B, Bangladesh; VN, Vietnam; T, Thailand.



observed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [Yamashita et al., 1998]. In one previous study, the comparison of nucleotide sequences between the German and Brazilian Aichi strains with the Japanese strains revealed the VP1 coding region to be the most variable of the structural protein domains [Oh et al., 2006]. Nucleotide identities in this region were lower (86–87%) than in the 3CD section (minimum 88%). That finding was in agreement with the presumption of Yamashita et al. [2000], who suggested that sequence diversity in the structural protein coding regions might be higher than in the 3CD region. In this region, as well as over the whole length of the ORF, the genotype A sequences were more closely related to each other than to that of the Brazilian type B isolate [Oh et al., 2006]. In this study, regarding the mean nucleotide distances among all Aichi virus strains of the three structural domains of the capsid gene, the VP1 domain showed the highest percentage of 15.2% when compared to those of the VP0 and VP3 domains (12.1% and 13.3%, respectively). This finding confirms that the VP1 domain is the most variable region of the capsid gene of Aichi virus.

In this study, full lengths of the capsid gene of 12 Aichi virus strains isolated in the four countries were successfully obtained. The phylogenetic tree constructed based on the capsid sequences of global Aichi strains including the strains studied and the reference strains showed that Aichi viruses could be divided into two major lineages: lineage I includes the Aichi virus strains isolated in Japan, Germany, Thailand, and Vietnam, lineage II comprises of Aichi strains from Bangladesh and Brazil. This proposal was also supported by the nucleotide distance variations up to more than 12.7% among Aichi strains between the two lineages (Table II). Lineage I could be further divided into four sub-lineages from a to d, in which, sub-lineage Ia consists of Japanese and Vietnamese strains in this study, sub-lineage Ib contains Japanese reference strains, sub-lineages Ic comprises of the German reference strain and one studied Japanese strain, and sub-lineage Id includes the studied Thai strain (Fig. 1 and Table II). In addition, three phylogenetic trees constructed from each of three nucleotide segments of the VP0, VP3, and VP1 domains were similar to one another and to the phylogenetic tree constructed from the whole capsid gene (data not shown). Taken together, the findings indicated that the VP1 domain is the most variable region of the capsid gene and appropriate for classification of Aichi viruses.

In comparison to the phylogenetic tree constructed from nucleotide sequences of the 3CD junction region in previous studies, it is noteworthy that all the Aichi virus strains of lineages I and II were clustered into the same genotypes A and B, respectively. Based on the 12 strains studied and the 5 reference strains, the phylogenetic tree constructed from the capsid gene was quite similar to that of the 3CD junction region, in which, the Japanese, Thai, and Vietnamese strains used in this study belonged to genotype A and the three Bangladeshi strains were included in genotype B [Yamashita et al.,

2000; Oh et al., 2006; Pham et al., 2007]. Therefore, the genetic differences between the two Aichi virus lineages seem to be not only due to the variation of the capsid gene, but also of the other regions of Aichi virus genome. However, in agreement with previous findings and suggestion, the sequence diversity of the capsid gene was higher than in the 3CD region. The nucleotide distances between Aichi virus strains of the two lineages I and II based on the capsid gene were equal to or higher than 12.7% (Table II), while those of the two genotypes A and B based on 3CD region were less than 10% (data not shown).

By Figure 2, the different amino acids between Aichi virus strains of lineages I and II could be found throughout the whole deduced amino acid sequences. Those amino acid substitutions which were considered to be lineage-specific, especially the different triple of amino acids at positions 220–222 located within the VP0 region, can be used as a useful reference to develop primers for genotyping. Also, it could be presumed that the different antigenicity between strains of the two lineages might attribute to the differences of all the three capsid proteins. This hypothesis should be elucidated in future studies on epitopes of capsid proteins of Aichi viruses.

Despite of the limited number of the strains used, this study may be useful in providing the classification of Aichi virus based on the capsid gene and contributing background data for future researches into Aichi viruses. Since Aichi virus is a new virus discovered recently, it should be recognized more widely in other countries, and further genetic research is necessary.

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## Association of RANTES –403 G/A, –28 C/G and In1.1 T/C Polymorphism With HIV-1 Transmission and Progression Among North Indians

Anurag Rathore,<sup>1</sup> Animesh Chatterjee,<sup>1</sup> P. Sivarama,<sup>2</sup> Naohiko Yamamoto,<sup>3</sup> Pradeep K. Singhal,<sup>4</sup> and Tapan N. Dhole<sup>1\*</sup>

<sup>1</sup>Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India

<sup>2</sup>AIDS Counseling and Treatment Center, Belgaum, India

<sup>3</sup>Department of International Health, Nagoya University School of Medicine, Nagoya, Japan

<sup>4</sup>Professional Biotech Pvt. Ltd., New Delhi, India

The relationships between host immune factors and HIV-1 disease progression are still in dispute. The RANTES SNPs exhibit distinct ethnic distribution and are associated with different effects on the course of HIV infection. Therefore, impact of RANTES gene polymorphism on HIV-1 transmission and progression needs to be evaluated. The RANTES genotypes were identified by PCR-RFLP method and confirmed by sequencing in HIV-1 seronegative (HSN;  $n=315$ ), HIV-1 exposed seronegative (HES;  $n=47$ ) and HIV-1 seropositive (HSP;  $n=196$ ) patients classified into different clinical stages (i.e. Stages I, II, III). Fisher exact test was used for statistical analysis and Arlequin software for haplotype analysis. RANTES allele –403G, –28C and In1.1 T were the predominant allele in the subject studied. HSP group have higher frequency of RANTES In1.1 T allele compared with HSN (91.32% vs. 86.19%;  $P=0.013$ ) and HES (91.32% vs. 78.72%;  $P=0.001$ ). Higher frequency of RANTES In1.1 C allele in Stage III was observed, compared with Stage I (14.28% vs. 6.39%) and was significantly associated with high risk ( $P=0.047$ , OR=2.439, C.I.=1.061–5.609). Haplotype II (ACT) was significantly higher in HSP compared with HSN (9.69% vs. 1.58%) and associated with high risk ( $P<0.001$ , OR=6.655, C.I.=2.443–18.132). There were no significant differences in RANTES –403 A/G and –28 C/G genotype and allele distribution in all the groups compared. Our results implicate that RANTES In1.1 T allele and haplotype II (ACT) may be a risk factor for HIV-1 transmission while RANTES In1.1 C allele may be risk factor for disease progression among North Indians. **J. Med. Virol.** 80: 1133–1141, 2008. © 2008 Wiley-Liss, Inc.

**KEY WORDS:** RANTES polymorphism; HIV-1; transmission; progression; risk factors

### INTRODUCTION

Among genes that influence human susceptibility to HIV infection or AIDS progression, chemokine-receptor and chemokine genes were extensively studied because of their role as HIV co-receptor or co-receptor competitor, respectively. RANTES (regulated upon activation, normal T cell expressed and secreted) has emerged as one of the most important co-receptor competitor molecule of the human immune system involved in the pathogenesis of HIV infection. RANTES is an endogenous ligand for CCR5, the key co-receptor for M-tropic HIV-1 strains commonly seen in early infection [Koning et al., 2003]. RANTES acts by blocking of the HIV envelope glycoprotein gp120 to CCR5 and by reducing surface levels of CCR5 co-receptor [Mack et al., 1998]. However, there is evidence that RANTES might promote the replication of some HIV strains, particularly at high concentrations [Kelly et al., 1998; Kinter et al., 1998]. With respect to RANTES, CD4 T cells from highly exposed-uninfected individuals produce increased amounts compared with random blood donor controls [Paxton et al., 1996; Furci et al., 1997; Suresh et al., 2007]. Whereas, immortalized

\*Correspondence to: Tapan N. Dhole, Professor, Department of Microbiology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Raebareilly Road, Lucknow 226014, India.  
E-mail: tapandhole@yahoo.co.in

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CD4 T cells from AIDS patients produce much less RANTES than those from long-term non-progressors [Saha et al., 1998; Paxton et al., 1999]. Moreover, defective CCR5 expression in CCR5  $\Delta$  32 homozygotes results in the downregulation of RANTES expression thus affecting the RANTES level [Samson et al., 1996; Clegg et al., 2000; Paxton et al., 2001].

The study of host genetic polymorphism studies implicating RANTES -403 G/A, RANTES -28 C/G in the promoter region and RANTES In1.1 T/C in the first intron region has provided important evidence that control of RANTES expression might influence the risk and outcome of HIV-1 infection. The RANTES -28G variant was shown to elevate promoter activity in vitro [Liu et al., 1999a; An et al., 2002] and is associated with a slower rate of CD4 cell depletion in HIV-1 infected Japanese patients [Liu et al., 1999a]. Although these results were not confirmed in subsequent studies in other ethnic groups in which the RANTES -28G frequency was lower than in the Japanese [Gonzalez et al., 2001; An et al., 2002; Zhao et al., 2004], McDermott et al. reported that patients with RANTES -28G showed a clear trend towards slower rate of CD4 cell depletion in HIV-1 infected European Americans [McDermott et al., 2000]. Recent reports have shown the protective effect of RANTES -28G on HIV disease progression in Thais [Wichukchinda et al., 2006] while, delayed AIDS progression in HIV-1 infected Japanese hemophiliacs [Koizumi et al., 2007]. With respects to RANTES -403A variants, HIV-1 infected European American with RANTES -403A were initially reported to progress more slowly to AIDS [McDermott et al., 2000]. However, subsequent studies with a larger sample size showed that RANTES -403A or RANTES In1.1 C, a RANTES intron polymorphism which is in a strong linkage disequilibrium with RANTES -403A and which negatively affects RANTES transcription in vitro, was reported to be associated with an increased rate of disease progression [Gonzalez et al., 2001; An et al., 2002]. Recent studies of other populations reported that -403G allele may be associated with increased susceptibility to HIV infection [Zhao et al., 2004] whereas, no such association of RANTES -403 A/G and -28C/G polymorphism could be established in HIV-1 infected Ugandans [Cooke et al., 2006], North Indians [Suresh et al., 2006], and Spanish populations [Vidal et al., 2006]. In vitro functional data, acquired using luciferase reporter assays in transfected jurkat cells, demonstrated that the RANTES In1.1C allele was associated with reduced RANTES expression thus contributing to the rapid progression of AIDS in European-Americans and African-Americans [An et al., 2002], however, the SNP was not found to influence disease progression in Japanese Hemophiliacs [Koizumi et al., 2007]. RANTES In1.1 C without RANTES -28G had an accelerating effect on HIV disease progression [Wichukchinda et al., 2006]. In contrast to previous studies, authors have recently reported that homozygosity for the In1.1C allele was associated with protection from death in HIV-1 positive individuals in Ugandans [Cooke et al., 2006].

HIV-1 infected individuals have widely different rates of disease progression and several SNPs are reported to be effective in different clinical stages of HIV/AIDS patients. Among North Indians, we recently reported that homozygous DC-SIGNR 5/5 showed significant reduced risk of HIV-1 infection [Rathore et al., 2008a] while repeat region polymorphism in the DC-SIGN neck domain does not effect risk of HIV-1 transmission [Rathore et al., 2008b]. Till date, only single study was conducted in which effect of RANTES genetic variants was assessed for HIV/AIDS transmission among North Indians [Suresh et al., 2006]. The global, ethnic and regional distribution of this HIV/AIDS protective mutations frequency varies significantly giving each population a different genetic resistance profile to the HIV infection and AIDS progression. Therefore, it is important to investigate factors modulating rates of disease progression for designing novel therapies and vaccines study.

## MATERIALS AND METHODS

### Patient Selection

One hundred and ninety-six HIV-1 seropositive patients (HSPs) were enrolled from the outpatients attending the clinics of Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India from January 2004 to December 2006. The HIV-1 seropositive subjects were in different stages of disease progression and had no history or a less than 6-week prior history of antiretroviral therapy. Depending on their CD4 counts and based on clinical symptoms, HIV-1 patients were divided into three subgroups, on the basis of disease severity, as per the Centers for disease control and prevention (CDC) criteria. Three groups were as follows: Stage I (n=86; asymptomatic HIV-1 patients in CDC category A1 to A3, Stage II (n=61; symptomatic HIV-1 patients without AIDS in CDC category B1 to B3), Stage III (n=49; symptomatic HIV-1 patients with AIDS in CDC category C1 to C3) [McNicholl et al., 2003; Vajpayee et al., 2005]. Only individuals who had a minimum follow-up time of at least 6 months were included in the study. 315 age-matched normal healthy controls of similar ethnicity with HIV-1 seronegative status (HSN) were recruited for the present study. 47 HIV-1 seronegative individuals with history of repeated sexual intercourse (twice a week) without any protection (e.g. condoms) with HIV-1 infected partners for at least 1-year were recruited in HIV-1 exposed seronegative group (HES). HIV-1 seronegative status of the HES subjects was confirmed by Western blot at the regular interval of 3 months till 1 year. Demographic profiles of the study groups are given in Table I. After an informed consent, 5-ml blood sample was taken in EDTA for analysis of DNA.

### Determination of HIV-1 Status and CD4 Cell Count

All individuals were screened for their HIV-1 status by primarily screening with ELISA (Vironostika, HIV Uni-FormII Ag/Ab, Biomerieux, Netherlands) and sub-



TABLE I. Demographic and Clinical Characteristics of the Study Groups

|  |              |
|--|--------------|
| HIV-1 seronegative (HSN; n = 315)        |              |
| Age (median, range)                      | 58, 35-60    |
| Sex (M/F ratio)                          | 6/1          |
| HIV-1 exposed seronegative (HES; n = 47) |              |
| Age (median, range)                      | 59, 35-65    |
| Sex (M/F ratio)                          | 7/1          |
| HIV-1 seropositive (HSP; n = 196)        |              |
| Age (median, range)                      | 57, 30-67    |
| Sex (M/F ratio)                          | 8/1          |
| CD4 counts/ $\mu$ l (median, range)      | 145, 30-1304 |
| Stage I <sup>a</sup> (n = 86)            |              |
| Age (median, range)                      | 58, 30-65    |
| Sex (M/F ratio)                          | 7/1          |
| CD4 counts/ $\mu$ l (median, range)      | 315, 65-1304 |
| Stage II <sup>a</sup> (n = 61)           |              |
| Age (median, range)                      | 55, 35-60    |
| Sex (M/F ratio)                          | 9/1          |
| CD4 counts/ $\mu$ l (median, range)      | 175, 60-512  |
| Stage III <sup>a</sup> (n = 49)          |              |
| Age (median, range)                      | 52, 38-67    |
| Sex (M/F ratio)                          | 8/1          |
| CD4 counts/ $\mu$ l (median, range)      | 120, 30-450  |

n: number of individuals.

<sup>a</sup>These groups were subgroups of the HIV-1 seropositive patient and were classified according to CDC (Centers for disease control and prevention) (23,24).

sequently confirmed with Western blot (LAV Blot I, BioRad, France). CD4 cell count was measured by flow cytometer FACScan (Becton Dickinson Immunocytometry systems, San Jose, CA) using fresh EDTA-treated whole blood at the time of recruitment.

#### Genomic DNA Isolation

The genomic DNA samples were obtained from 0.2 to 0.3 ml peripheral whole blood by using QIAamp Blood kit (Qiagen, CA) according to the protocols supplied by the manufacturer. Usually ~0.1  $\mu$ g genomic DNA was used for the genotyping studies.

#### Polymerase Chain Reaction and Sequencing

RANTES -403G/A genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method as described previously [Liu et al., 1999b]. Amplification of a 124 bp fragment spanning position -403 was done by PCR using forward primer 5'-taa cat cct tcc atg gat cag gg-3' and reverse primer 5'-tgg cag tta gga cag gat-3'. PCR was carried out in 25  $\mu$ l reaction volume containing 0.1  $\mu$ M each primer, 100  $\mu$ M dNTPs, 2.5 mM MgCl<sub>2</sub>, and 0.5 unit of Taq DNA polymerase (Roche, Germany) in 1 $\times$  buffer supplied with enzyme. The reaction mixture was subjected to 40 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 3 sec. Digestion with MnlI (Fermentas) yielded a 103 bp and a 21 bp fragment only when G was present at position -403 and was determined by electrophoresis in 20% polyacrylamide gel.

RANTES promoter -28C/G was genotyped by PCR-RFLP as described previously [Liu et al., 1999b]. Briefly, amplification of 136 bp fragment spanning position -28 was amplified from extracted genomic DNA using forward primer 5'-ccg gag gct att tca gtt tt-3' and reverse

primer 5'-gca gct cag gct ggc cct t-3' with same PCR conditions as mentioned above. PCR was carried out in 25  $\mu$ l reaction volume containing 0.1  $\mu$ M each primer, 100  $\mu$ M dNTPs, 2.5 mM MgCl<sub>2</sub>, and 0.5 unit of Taq DNA polymerase (Roche, Germany) in 1 $\times$  buffer supplied with enzyme. The presence of a MnlI site yielded an 111 bp and 25 bp fragment only when C was present at position -28, and was determined by electrophoresis in 20% polyacrylamide gel.

Genotyping of RANTES In1.1C was carried out by previously described protocol [Ahlenstiel et al., 2005] using forward primer: 5'-CCT GGT CTT GAC CAC CAC A-3' and reverse primer: 5'-GCT GAC AGG CAT GAG TCA GA-3'. A 343-bp fragment spanning position In1.1C was amplified from extracted genomic DNA. Amplification was done for 40 cycles at 94°C for 1 sec, 55°C for 40 sec, and 72°C for 1 min. PCR was carried out in 25  $\mu$ l reaction volume containing 0.1  $\mu$ M each primer, 100  $\mu$ M dNTPs, 2.5 mM MgCl<sub>2</sub>, and 0.5 unit of Taq DNA polymerase (Roche, Germany) in 1 $\times$  buffer supplied with enzyme. The presence of MboII (Fermentas) site yielded a 234 and 109 bp corresponding to the presence of a C at position RANTES In1.1, and was determined by electrophoresis in 2.5% agarose gel. All the PCR-amplified products were randomly selected to be cloned and sequenced to verify the PCR-RFLP results according to the manufacturers protocols (Applied Biosystems/Perkin Elmer Foster City, CA) using the Dye Terminator cycle sequencing kit in ABI PRISM 310 genetic Analyzer.

#### Statistical Analysis

Statistical analysis was done by SPSS software version 11.5 (SPSS, Chicago, IL). Direct gene counting method was used to determine the frequency of genotypes and alleles. The Fisher's exact or  $\chi^2$  test was used to determine differences in allele/genotype frequencies of RANTES genetic variants. Odds ratios (OR) and its 95% confidence interval (C.I.) were obtained to describe the strength of association. Observed and expected genotype frequencies were compared by  $\chi^2$  test to check deviation from Hardy Weinberg Equilibrium. A *P* value <0.05 was considered to be statistically significant. The haplotype frequencies were calculated from the observed genotypes by using the Arlequin software vers. 2.000 [Schneider et al., 2000].

#### RESULTS

We genotyped 315 HIV-1 seronegative (HSN), 47 HIV-1 exposed seronegative (HES) and 196 HIV-1 seropositive (HSP) patient for RANTES -403A/G, -28C/G and In1.1T/C polymorphism. Genotype distribution and allelic frequency were compared between HSN, HES and HSP and also between three different subgroups (Stages I, II, III) of HSP group. The distributions of the genotypic frequencies of the RANTES polymorphism were in Hardy-Weinberg equilibrium. The percent allele/genotype frequencies corresponding to three SNPs are shown in Tables II and III.

TABLE II. Genotype and Allele Frequencies of RANTES -403 G/A, -28 C/G, In.1.1 T/C Polymorphism Among HIV-1 Seropositive and HIV-1 Exposed Seronegative Individuals

| Genotype          | HSP<br>(n = 196) | HSN<br>(n = 315) | Odds ratio | P value | Confidence interval | HES (n = 47) | Odds ratio <sup>a</sup> | P value <sup>a</sup> | Confidence interval <sup>a</sup> |
|-------------------|------------------|------------------|------------|---------|---------------------|--------------|-------------------------|----------------------|----------------------------------|
| RANTES -403 A/G   |                  |                  |            |         |                     |              |                         |                      |                                  |
| G                 | 319(81.37)       | 534(84.76)       | 0.786      | 0.166   | 0.562-1.098         | 72(76.59)    | 1.335                   | 0.311                | 0.777-2.293                      |
| A                 | 73(18.623)       | 96(15.23)        | 1.273      | 0.166   | 0.911-1.779         | 22(23.40)    | 0.749                   | 0.311                | 0.436-1.286                      |
| GG                | 132(67.34)       | 230(73.01)       | 0.762      | 0.193   | 0.517-1.124         | 27(57.44)    | 1.528                   | 0.233                | 0.797-2.929                      |
| GA                | 55(28.06)        | 74(23.49)        | 1.270      | 0.251   | 0.846-1.907         | 18(38.29)    | 0.628                   | 0.214                | 0.323-1.223                      |
| AA                | 9(4.59)          | 11(3.49)         | 1.330      | 0.640   | 0.541-3.270         | 2(4.25)      | 1.083                   | 1.000                | 0.226-5.186                      |
| RANTES -28 C/G    |                  |                  |            |         |                     |              |                         |                      |                                  |
| C                 | 388(98.97)       | 617(97.93)       | 2.044      | 0.314   | 0.662-6.313         | 91(96.80)    | 3.198                   | 0.135                | 0.703-14.537                     |
| G                 | 4(1.02)          | 13(2.06)         | 0.489      | 0.314   | 0.158-1.511         | 3(3.19)      | 3.135                   | 0.135                | 0.069-1.422                      |
| CC                | 192(97.95)       | 302(95.87)       | 2.066      | 0.310   | 0.664-6.429         | 44(93.61)    | 3.273                   | 0.134                | 0.707-15.150                     |
| CG                | 4(2.04)          | 13(4.12)         | 0.484      | 0.310   | 0.156-1.506         | 3(6.38)      | 0.306                   | 0.134                | 0.066-1.414                      |
| GG                | 0                | 0                | —          | —       | —                   | 0            | —                       | —                    | —                                |
| RANTES In.1.1 T/C |                  |                  |            |         |                     |              |                         |                      |                                  |
| T                 | 358(91.32)       | 543(86.19)       | 1.687      | 0.013   | 1.110-2.563         | 74(78.72)    | 2.846                   | 0.001                | 1.552-5.219                      |
| C                 | 34(8.67)         | 87(13.80)        | 0.593      | 0.013   | 0.390-0.901         | 20(21.27)    | 0.351                   | 0.001                | 0.192-0.644                      |
| TT                | 166(84.69)       | 230(73.01)       | 2.045      | 0.002   | 1.289-3.245         | 28(59.5)     | 3.755                   | <0.001               | 1.864-7.564                      |
| TC                | 28(13.26)        | 83(26.34)        | 0.427      | <0.001  | 0.264-0.693         | 18(38.2)     | 0.246                   | <0.001               | 0.120-0.505                      |
| CC                | 4(2.04)          | 2(0.63)          | 3.260      | 0.210   | 0.592-17.970        | 1(2.12)      | 0.958                   | 1.000                | 0.105-8.778                      |

HSP, HIV-1 seropositive; HSN, HIV-1 seronegative; HES, HIV-1 exposed seronegative.  
Number in parentheses gives the data in percentage.

<sup>a</sup>HSP vs. HES.

TABLE III. Genotype and Allele Frequencies of RANTES -403 G/A, -28 C/G, In.1.1 T/C Polymorphism Among HIV-1 Seropositive Patients Classified into Stages I, II and III

| Genotype          | Stage III (n = 49) | Stage I (n = 86) | Stage II (n = 61) | Odds ratio <sup>a</sup> | P value | Confidence interval | Odds ratio <sup>a</sup> | P value <sup>a</sup> | Confidence interval <sup>a</sup> |
|-------------------|--------------------|------------------|-------------------|-------------------------|---------|---------------------|-------------------------|----------------------|----------------------------------|
| RANTES -403 A/G   |                    |                  |                   |                         |         |                     |                         |                      |                                  |
| G                 | 75(76.53)          | 148(86.04)       | 96(78.68)         | 0.529                   | 0.065   | 0.280-0.999         | 0.883                   | 0.746                | 0.467-1.670                      |
| A                 | 23(23.46)          | 24(13.95)        | 26(21.31)         | 1.891                   | 0.065   | 1.001-3.571         | 1.132                   | 0.746                | 0.599-2.141                      |
| GG                | 30(61.22)          | 63(73.25)        | 39(63.93)         | 0.576                   | 0.177   | 0.273-1.217         | 0.891                   | 0.844                | 0.410-1.937                      |
| GA                | 15(30.61)          | 22(25.58)        | 18(29.50)         | 1.283                   | 0.552   | 0.590-2.791         | 1.054                   | 1.000                | 0.464-2.392                      |
| AA                | 4(8.16)            | 1(1.16)          | 4(6.55)           | 7.556                   | 0.058   | 0.820-69.629        | 1.267                   | 1.000                | 0.300-5.346                      |
| RANTES -28 C/G    |                    |                  |                   |                         |         |                     |                         |                      |                                  |
| C                 | 97(98.97)          | 171(99.41)       | 120(98.36)        | 0.567                   | 1.000   | 0.035-9.171         | 1.617                   | 1.000                | 0.144-18.096                     |
| G                 | 1(1.02)            | 1(0.58)          | 2(1.63)           | 1.763                   | 1.000   | 0.109-28.501        | 0.619                   | 1.000                | 0.055-6.924                      |
| CC                | 48(97.95)          | 85(98.83)        | 59(96.72)         | 0.565                   | 1.000   | 0.035-9.234         | 1.627                   | 1.000                | 0.143-18.491                     |
| CG                | 1(2.04)            | 1(1.16)          | 2(3.27)           | 1.771                   | 1.000   | 0.108-28.955        | 0.615                   | 1.000                | 0.054-6.984                      |
| GG                | 0                  | 0                | 0                 | —                       | —       | —                   | —                       | —                    | —                                |
| RANTES In.1.1 T/C |                    |                  |                   |                         |         |                     |                         |                      |                                  |
| T                 | 84(85.71)          | 161(93.60)       | 113(92.62)        | 0.410                   | 0.047   | 0.178-0.943         | 0.478                   | 0.121                | 0.197-1.156                      |
| C                 | 14(14.28)          | 11(6.39)         | 9(7.37)           | 2.439                   | 0.047   | 1.061-5.609         | 2.093                   | 0.121                | 0.865-5.064                      |
| TT                | 36(73.46)          | 76(88.37)        | 54(88.52)         | 0.364                   | 0.033   | 0.146-0.910         | 0.359                   | 0.050                | 0.131-0.987                      |
| TC                | 12(24.48)          | 9(10.46)         | 5(8.19)           | 2.775                   | 0.047   | 1.074-7.168         | 3.632                   | 0.032                | 1.182-11.165                     |
| CC                | 1(2.04)            | 1(1.16)          | 2(3.27)           | 1.771                   | 1.000   | 0.108-28.955        | 0.615                   | 1.000                | 0.054-6.984                      |

Number in parentheses gives the data in percentage.  
<sup>a</sup>Stage III vs. Stage II.