RANTES Allele/Genotype Distribution Analysis

The frequency of RANTES In1.1 T allele was significantly higher in HSP compared with HSN (91.32% vs. 86.19%) and HES (91.32% vs. 78.72%) and associated with high risk ($P = 0.013$, OR = 1.687, C.I. = 1.110–2.563 and $P = 0.001$, OR = 2.846, C.I. = 1.552–5.219), respectively. The frequency of RANTES 1.1 TT genotype was significantly higher in HSP compared with HSN (84.69% vs. 73.01%) and HES (84.69% vs. 59.57%) and associated with high risk ($P = 0.002$, OR = 2.045, C.I. = 1.289–3.245 and $P < 0.001$, OR = 3.755, C.I. = 1.864–7.564), respectively (Table II).

A further comparison in between different stages of HIV-1 infected patients revealed higher frequency of RANTES 1.1 C allele in Stage III 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). The frequency of RANTES 1.1 TC genotype was higher in Stage III compared with Stage I (24.48% vs. 10.46%) and II (24.48% vs. 8.19%) and was significantly associated with high risk ($P = 0.047$, OR = 2.775, C.I. = 1.074–7.168) and ($P = 0.032$, OR = 3.632, C.I = 1.182–11.165), respectively. RANTES −403A/G and −28G/C allele or genotype frequencies were similar in all the compared groups (Table III).

RANTES Haplotype Analysis

Four haplotype I (GCT), II (ACT), III (ACC) and IV (AGC) were detected in North Indian individual (Tables IV and V). Frequency of haplotype I (GCT) was found to be predominant and fairly similar in all the studied groups. Haplotype II (ACT) was found at significantly higher frequency in HSP compared with HSN (9.69% vs. 1.58%) and was associated with high risk ($P < 0.001$, OR = 6.655, C.I. = 2.443–18.132). Though, frequency of haplotype II (ACT) was higher in HSP compared with HES (9.69% vs. 2.12%) but was not significantly associated with high risk ($P = 0.136$, OR = 4.938, C.I. = 0.644–37.857) (Table IV). Frequency of haplotype III (ACC) was though higher in Stage III compared with Stage I (12.24% vs. 4.65%; $P = 0.169$, OR = 2.86, C.I. = 0.766–10.685) and Stage II (12.24% vs. 6.55%; $P = 0.336$, OR = 1.988, C.I. = 0.528–7.485), but was not significant (Table V). The minor haplotype observed in the study was haplotype IV (AGC), but there was no significant difference in its frequency in the healthy controls versus HIV-1-infected study subjects.

Further stratification of the groups by age and gender did not provide evidence of an association between RANTES alleles or genotypes and HIV infection (data not shown).

DISCUSSION

Several single nucleotide polymorphisms in the RANTES gene have been reported to influence the natural course of HIV infection by up or down-regulating RANTES gene activity. The most frequent of those polymorphic sites comprise −28 C/G and −403 G/A in the promoter region and In1.1 T/C in the first intron

TABLE IV. Haplotype Frequencies of RANTES 403/28/In1.1 Polymorphism Among HIV-1 Seropositive, HIV-1 Seronegative and HIV-1 Exposed Seronegative Individuals

Haplotype (403/28/In1.1)	HSP (n = 196)	HSN (n = 315)	Odds ratio	P value	Confidence interval	HES (n = 47)	Odds ratio[a]	P value[a]	Confidence interval[a]
Haplotype I GCT	160(81.63)	267(84.76)	0.799	0.391	0.497–1.284	36(76.59)	1.358	0.418	0.631–2.921
Haplotype II ACT	19(9.69)	5(1.58)	6.655	<0.001	2.443–18.132	1(2.12)	4.938	0.136	0.644–37.857
Haplotype III ACC	15(7.65)	37(11.74)	0.623	0.175	0.332–1.167	8(17.02)	0.404	0.091	0.160–1.019
Haplotype IV AGC	2(1.02)	6(1.90)	0.531	0.717	0.106–2.657	2(4.25)	0.232	0.169	0.032–1.691

HSP: HIV-1 seropositive; HSN: HIV-1 seronegative; HES: HIV-1 exposed seronegative.
Number in parentheses gives the data in percentage.
[a]HSP vs. HES.

TABLE V. Haplotype Frequencies of RANTES 403/28/In1.1 Polymorphism Among HIV-1 Seropositive Patients Classified into Stages I, II and III

Haplotype (403/28/In1.1)	Stage III (n = 49)	Stage I (n = 86)	Odds ratio	P value	Confidence interval	Stage II (n = 61)	Odds ratio[a]	P value[a]	Confidence interval[a]
Haplotype I GCT	37(75.51)	74(86.04)	0.500	0.160	0.205–1.220	48(78.68)	0.835	0.820	0.342–2.042
Haplotype II ACT	5(10.20)	7(8.13)	1.282	0.757	0.384–4.281	8(13.11)	0.753	0.770	0.230–2.466
Haplotype III ACC	6(12.24)	4(4.65)	2.860	0.169	0.766–10.685	4(6.55)	1.988	0.336	0.528–7.485
Haplotype IV AGC	1(2.04)	1(1.16)	1.771	1.000	0.108–28.955	1(1.637)	1.250	1.000	0.076–20.508

Number in parentheses gives the data in percentage.
[a]Stage III vs. Stage II.

region of RANTES gene. The −28G and −403A variant were reported to up-regulate RANTES transcription [Liu et al., 1999a; Nickel et al., 2000], while In1.1C allele was reported to decrease RANTES transcriptional activity thus reducing the gene expression [An et al., 2002].

This is the first host-genetic polymorphism study with a substantial sample size in the North Indians, addressing an effect of RANTES genetic variants on HIV-1 disease progression. The frequency of the RANTES −403 A and −28 G allele at the promoter region was 15.23% and 2.06% respectively in the North Indian general population. The frequency was the lowest compared with those in other populations studied (Table VI). The frequency of In1.1 C allele was 13.80% similar to Caucasians and European American, but lower than Han Chinese Asians, Ugandans and African American suggesting the ethnic variability in the allelic frequencies of this gene. Because of this variation in gene frequencies between different ethnic groups it is crucial to repeat genetic association studies in each group rather than to generalize these findings when we try to establish the relationship between a polymorphic marker and HIV/AIDS susceptibility or progression. The most common haplotype observed in this study was haplotype I (GCT), which is in concordant with previous studies [An et al., 2002; Wichukchinda et al., 2006].

A relative comparison between HSN, HES and HSP subjects was carried out to assess the effect of RANTES variability on risk of HIV transmission/susceptibility. The results obtained in this study are consistent with previous studies on HIV-1 exposed but seronegative North Indian individuals [Suresh et al., 2006] and Whites individuals [Liu et al., 2004], that showed no correlation of RANTES −403A/G and −28G/C polymorphism with susceptibility against HIV-1 infection. Cross-sectional genetic association case–control study on White Spaniards population [Vidal et al., 2006] and Japanese [Liu et al., 1999a] also revealed no association of −403 A/G and −28G/C polymorphism with HIV-1 susceptibility. In contrast, 403A allele and wild-type 403 G allele increases susceptibility to HIV-1 infection in white Americans [McDermott et al., 2000; An et al., 2002], Han Chinese [Liu et al., 2003] and in Chinese subjects [Zhao et al., 2004], respectively. Furthermore, RANTES In.1.1 C allele was reported to be associated with increased susceptibility to HIV-1 infection in US cohorts of HIV-1 positive patients [An et al., 2002]. In contrast, present study reports that RANTES In1.1 T allele was found at a higher frequency in the HSP group compared with HSN and HES suggesting that the RANTES 1.1 T allele may be associated with increased susceptibility to HIV-1 transmission. To approve these results, we use the haplotype analysis to test association of this polymorphism with HIV-1 susceptibility. Haplotype II (ACT) that corresponds to the haplogroup R2 in other studies [An et al., 2002] was found to be overrepresented in HIV-1 seropositive individuals compared to HIV-1 seronegative though statistical significant association was not observed when compared with

TABLE VI. Frequency of RANTES −403 G/A, −28 C/G, and In1.1 T/C Alleles in North Indian and Other Ethnic Population

Populations	Samples	RANTES −403G (%)	RANTES −403A (%)	RANTES −28C (%)	RANTES −28G (%)	RANTES In1.1 T (%)	RANTES In1.1 C (%)	Reference
North Indians	315	84.76	15.23	97.93	2.06	86.19	13.80	Present study
African American	1,574	57.00	43.00	Very high	Very low	80.00	20.00	Paxton et al. [2001]
Caucasians	109	84.86	15.13	97.20	2.70	88.99	11.00	Liu et al. [1999]
Chinese	439	62.30	37.70	87.70	12.30	N.D.	N.D.	Zhao et al. [2004]
European Americans	2,594	82.00	18.00	Very high	Very low	86.00	14.00	Paxton et al. [2001]
Han Chinese Asian	129	64.00	36.00	87.00	13.00	66.00	34.00	Paxton et al. [2001]
Japanese	193	62.40	37.56	83.40	16.50	N.D.	N.D.	Liu et al. [1999]
North Indians	75	88.00	12.00	99.00	1.00	N.D.	N.D.	Suresh et al. [2006]
Thai Females	119	73.10	26.90	92.40	7.60	76.50	23.50	Wichukchinda et al. [2006]
Ugandans	94	53.00	47.00	N.D.	N.D.	80.00	20.00	Cooke et al. [2006]
White Spaniards	100	82.00	18.00	97.00	3.00	N.D.	N.D.	Vidal et al. [2006]

N.D.: not detected.

HIV-1 exposed seronegative. But, when HIV-1 seropositive group was compared with HIV-1 exposed seronegative and HIV-1 seronegative taken together significant association was found suggesting its possible role in HIV-1 acquisition. Similarly, another RANTES haplotype III (ACC) in this study can be compared with the haplotype R4 that has been shown to be associated with increased risk of infection in European Americans [An et al., 2002], but not in presently studied Indian individuals. Possible explanation for discrepancy between previous and present study may be because influence of the allelic variants of RANTES gene on the risk of HIV-1 susceptibility varied widely and depended on the origin of the subjects analyzed.

HIV-1 seropositive patients classified into different stages of HIV/AIDS disease (Stages I, II and III) were analyzed to evaluate their effect on development of AIDS. However, one limitation of this study is that the HIV-1 positive patients could not be classified on the basis of number of years of known seroprevalence without antiretroviral therapy and viral load. Nonetheless, in the absence of such a longitudinal follow up data, the patients were categorized on the basis of their CD4 counts and clinical CDC classification. RANTES In1.1 C allele was found at a higher frequency in the patients classified as CDC Stage III compared with Stage I, suggesting its possible role in development of AIDS. Our results were in agreement with the previous studies that reported, RANTES In1.1 C contribute to the rapid progression and had an accelerating effect on HIV disease progression in European Americans, African Americans [An et al., 2002] and HIV-infected Thais [Wichukchinda et al., 2006]. None of the haplotypes were found to be associated with disease progression among North Indian HIV-1 infected patients, though previous studies have showed haplotype III (ACC) which correspond to haplogroup R3, accelerated AIDS progression in both European American and African American [An et al., 2002]. The lack or the presence of association in HIV-1 susceptibility/progression between previous and present study may reflect genetic heterogeneity in the pathogenesis of HIV/AIDS. Different risk alleles may be important in different populations. Discrepancies between our results and the previous studies may be due to differences in ethnicity, environmental and social conditions of the population. Moreover, differences in design and recruitment of subjects between this study and previous genetic studies cannot be ignored.

In conclusion, we have studied genetic polymorphism known to be involved in the risk of HIV infection and the rate of HIV disease progression. We have shown that, with respect to HIV-1 susceptibility RANTES In1.1 TT genotype/T allele and haplotype ACT are major risk factors for HIV-1 transmission while RANTES In1.1 C allele is a risk factor for accelerating disease progression among North Indians. Further genetic studies covering more number of SNPs in the RANTES and neighboring region tested in a larger number of well-categorized seropositive subjects are warranted.

ACKNOWLEDGMENTS

We gratefully thank Mr. S. Pandey, V. K. Mishra, Department of Microbiology, SGPGIMS, Lucknow for assistance in coordinating the clinical samples and all of the individuals who participated in the study. The Senior Research Fellowship provided by Indian Council of Medical Research (ICMR, New Delhi) to Mr. Anurag Rathore (first author) is greatly acknowledged.

REFERENCES

Ahlenstiel G, Iwan A, Nattermann J, Bueren K, Rockstroh JK, Brackmann HH, Kupfer B, Landt O, Peled A, Sauerbruch T, Spengler U, Woitas RP. 2005. Distribution and effects of polymorphic RANTES gene alleles in HIV/HCV coinfection—a prospective cross-sectional study. World J Gastroenterol 11:7631–7638.

An P, Nelson GW, Wang L, Donfield S, Goedert JJ, Phair J, Vlahov D, Buchbinder S, Farrar WL, Modi W, O'Brien SJ, Winkler CA. 2002. Modulating influence on HIV/AIDS by interacting RANTES gene variants. Proc Natl Acad Sci USA 99:10002–10007.

Clegg AO, Ashton LJ, Biti RA, Badhwar P, Williamson P, Kaldor JM, Stewart GJ. 2000. CCR5 promoter polymorphisms, CCR5 59029A and CCR5 59353C, are under represented in HIV-1-infected long-term non-progressors. The Australian Long-Term Non-Progressor Study Group. AIDS 14:103–108.

Cooke GS, Tosh K, Ramaley PA, Kaleebu P, Zhuang J, Nakiyingi JS, Watera C, Gilks CF, French N, Whitworth JA, Hill AV. 2006 A polymorphism that reduces RANTES expression is associated with protection from death in HIV-seropositive Ugandans with advanced disease. J Infect Dis 194:666–669.

Furci L, Scarlatti G, Burastero S, Tambussi G, Colognesi C, Quillent C, Longhi R, Loverro P, Borgonovo B, Gaffi D, Carrow E, Malnati M, Lusso P, Siccardi AG, Lazzarin A, Beretta A. 1997. Antigen-driven C-C chemokine-mediated HIV-1 suppression by CD4 (+) T cells from exposed uninfected individuals expressing the wild-type CCR-5 allele. J Exp Med 186:455–460.

Gonzalez E, Dhanda R, Bamshad M, Mummidi S, Geevarghese R, Catano G, Anderson SA, Walter EA, Stephan KT, Hammer MF, Mangano A, Sen L, Clark RA, Ahuja SS, Dolan MJ, Ahuja SK. 2001. Global survey of genetic variation in CCR5, RANTES, and MIP-1alpha: impact on the epidemiology of the HIV-1 pandemic. Proc Natl Acad Sci USA 98:5199–5204.

Kelly MD, Naif HM, Adams SL, Cunningham AL, Lloyd AR. 1998. Dichotomous effects of beta-chemokines on HIV replication in monocytes and monocyte-derived macrophages. J Immunol 160: 3091–3095.

Kinter A, Catanzaro A, Monaco J., et al. 1998. CC-chemokine enhance the replication of T-tropic strains of HIV-1 in CD4 (+) T cells: role of signal transduction. Proc Natl Acad Sci USA 95:11880–11885.

Koizumi Y, Kageyama S, Fujiyama Y, Miyashita M, Lwembe R, Ogino K, Shioda T, Ichimura H. 2007. RANTES −28G delays and DC-SIGN - 139C enhances AIDS progression in HIV type 1-infected Japanese hemophiliacs. AIDS Res Hum Retroviruses 23:713–719.

Koning FA, Kwa D, Boeser-Nunnick B., et al. 2003. Decreasing sensitivity to RANTES neutralization of CC chemokine receptor-5 using non-syncytium-inducing virus variants in the course of human immunodeficiency virus type 1 infection. J Infect Dis 188: 864–872.

Liu H, Chao D, Nakayama EE, Taguchi H, Goto M, Xin X, Takamatsu JK, Saito H, Ishikawa Y, Akaza T, Juji T, Takebe Y, Ohishi T, Fukutake K, Maruyama Y, Yashiki S, Sonoda S, Nakamura T, Nagai Y, Iwamoto A, Shioda T. 1999a. Polymorphism in RANTES chemokine promoter affects HIV-1 disease progression. Proc Natl Acad Sci USA 96:4581–4585.

Liu H, Hwangbo Y, Holte S, Lee J, Wang C, Kaupp N, Zhu H, Celum C, Corey L, McElrath MJ, Zhu T. 2004. Analysis of genetic polymorphisms in CCR5, CCR2, stromal cell-derived factor-1, RANTES, and dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin in seronegative individuals repeatedly exposed to HIV-1. JID 190:1055–1058.

Liu H, Shioda T, Nagai Y, Iwamoto A, Wasi C, Ma J, Liang W, Takebe Y, Theodorou I, Magierowska M, Krishnamoorty R, Chaventre A, Debre P. 1999b. Distribution of HIV-1 disease modifying regulated on activation normal T cell expressed and secreted haplotypes in Asian, African and Caucasian individuals. French ALT and IMMUNOCO Study Group. AIDS 13:2602–2603.

Liu XL, Wang FS, Jin L, Liu MX, Xu DZ. 2003. Preliminary study on the association of chemokine RANTES gene polymorphisms with HIV-1 infection in Chinese Han population. Zhonghua Liu Xing Bing Xue Za Zhi 24:971–975. Chinese.

Mack M, Luckow B, Nelson PJ, Cihak J, Simmons G, Clapham PR, Signoret N, Marsh M, Stangassinger M, Borlat F, Wells TN, Schlondorff D, Proudfoot AE. 1998. Aminooxypentane-RANTES induces CCR5 internalization but inhibits recycling: a novel inhibitory mechanism of HIV infectivity. J Exp Med 187:1215–1224.

McDermott DH, Beecroft MJ, Kleeberger CA, Al-Sharif FM, Ollier WE, Zimmerman PA, Boatin BA, Leitman SF, Detels R, Hajeer AH, Murphy PM. 2000. Chemokine RANTES promoter polymorphism affects risk of both HIV infection and disease progression in the Multicenter AIDS Cohort Study. AIDS 14:2671–2678.

McNicholl JM, Lal RB, Kaslow R. 2003. Human immunodeficiency virus (HIV) infection. In Cooper DN, (ed.): Encyclopedia of the Human Genome. Nature Publishing Group. www.ehgonline.net.

Nickel RG, Casolaro V, Wahn U, Beyer K, Barnes KC, Plunkett BS, Freidhoff LR, Sengler C, Plitt JR, Schleimer RP, Caraballo L, Naidu RP, Levett PN, Beaty TH, Huang SK. 2000. Atopic dermatitis is associated with a functional mutation in the promoter of the C-C chemokine RANTES. J Immunol 164:1612–1616.

Paxton WA, Kang S, Liu R, Landau NR, Gingeras TR, Wu L, Mackay CR, Koup RA. 1999. HIV-1 infectability of CD4+ lymphocytes with relation to beta-chemokines and the CCR5 coreceptor. Immunol Lett 66:71–75.

Paxton WA, Martin SR, Tse D, O'Brien TR, Skurnick J, VanDevanter NL, Padian N, Braun JF, Kotler DP, Wolinsky SM, Koup RA. 1996. Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposure. Nat Med 2:412–417.

Paxton WA, Neumann AU, Kang S, Deutch L, Brown RC, Koup RA, Wolinsky SM. 2001. RANTES production from CD4+ lymphocytes correlates with host genotype and rates of human immunodeficiency virus type 1 disease progression. J Infect Dis 183:1678–1681.

Rathore A, Chatterjee A, Sivarama P, Yamamoto N, Dhole TN. 2008a. Role of homozygous DC-SIGNR 5/5 tandem repeat polymorphism in HIV-1 exposed seronegative North Indian individuals. J Clin Immunol 28:50–57.

Rathore A, Chatterjee A, Sood V, Khan SZ, Banerjea AC, Yamamoto N, Dhole TN. 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. Clin Chim Acta 2008b. Jan 12; [Epub ahead of print].

Saha K, Bentsman G, Chess L, Volsky DJ. 1998. Endogenous production of beta-chemokines by CD4+, but not CD8+, T-cell clones correlates with the clinical state of human immunodeficiency virus type 1 (HIV-1)-infected individuals and may be responsible for blocking infection with non-syncytium-inducing HIV-1 in vitro. J Virol 72:876–881.

Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, Farber CM, Saragosti S, Lapoumeroulie C, Cognaux J, Forceille C, Muyldermans G, Verhofstede C, Burtonboy G, Georges M, Imai T, Rana S, Yi Y, Smyth RJ, Collman RG, Doms RW, Vassart G, Parmentier M. 1996. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nature 382:722–725.

Schneider S, Roessli D, Excoffier L. Arlequin ver 2.00. A software for population genetics data analysis. Switzerland: Genetics and Biometry Laboratory. University of Geneva. 2000.

Suresh P, Wanchu A, Bhatnagar A, Sachdeva RK, Sharma M. 2007. Spontaneous and antigen-induced chemokine production in exposed but uninfected partners of HIV type 1-infected individuals in North India. AIDS Res Hum Retroviruses 23:261–268.

Suresh P, Wanchu A, Sachdeva RK, Bhatnagar A. 2006. Gene polymorphisms in CCR5, CCR2, CX3CR1, SDF-1 and RANTES in exposed but uninfected partners of HIV-1 infected individuals in North India. J Clin Immunol 26:476–484.

Vajpayee M, Kaushik S, Sreenivas V, Wig N, Seth P. 2005. CDC staging based on absolute CD4 count and CD4 percentage in an HIV-1 infected Indian population: treatment implication. Clin Exp Immunol 141:485.

Vidal F, Peraire J, Domingo P, Broch M, Cairo M, Pedrol E, Montero M, Vilades C, Gutierrez C, Sambeat MA, Fontanet A, Dalmau D, Deig E, Knobel H, Sirvent JJ, Richart C, Veloso S, Saumoy M,

Lopez-Dupla M, Olona M, Cadafalch J, Fuster M, Ochoa A, Soler A, Guelar A, Gonzalez J. 2006. Chemokines and Long-Term Nonprogressive HIV01 Infection Study Group. Polymorphism of RANTES chemokine gene promoter is not associated with long-term nonprogressive HIV-1 infection of more than 16 years. J Acquir Immune Defic Syndr 41:17–22.

Wichukchinda N, Nakayama EE, Rojanawiwat A, Pathipvanich P, Auwanit W, Vongsheree S, Ariyoshi K, Sawanpanyalert P, Shioda T. 2006. Protective effects of IL4-589T and RANTES −28G on HIV-1 disease progression in infected Thai females. AIDS 20:189–196.

Zhao XY, Lee SS, Wong KH, Chan KC, Ma S, Yam WC, Yuen KY, Ng MH, Zheng BJ. 2004. Effects of single nucleotide polymorphisms in the RANTES promoter region in healthy and HIV-infected indigenous Chinese. Eur J Immunogenet 31:179–183.

cause this technology is available in relatively few clinical laboratories, cases of infection with *M. massiliense* may be mistakenly attributed to *M. abscessus*. Although infections with *M. massiliense* may be underrecognized, reports of these infections are raising concern. The capacity of this bacteria to infect different body sites is further evidence for the pathogenic potential of a rapidly growing mycobacteria in human infections (*10*).

Enrico Tortoli,* Rita Gabini,† Irene Galanti,† and Alessandro Mariottini*

*Careggi Hospital, Florence, Italy; and †San Donato Hospital, Arezzo, Italy

References

1. Böddinghaus B, Rogall T, Flohr T, Blöcker H, Böttger EC. Detection and identification of mycobacteria by amplification of rRNA. J Clin Microbiol. 1990;28:1751–9.
2. Adékambi T, Colson P, Drancourt M. *rpoB*-based identification of nonpigmented and late-pigmenting rapidly growing mycobacteria. J Clin Microbiol. 2003;41:5699–708.
3. Delaney V, Sumrani N, Hong JH, Sommer B. Mycobacterial infections in renal allograft recipients. Transplant Proc. 1993;25:2288–9.
4. Branger B, Gouby A, Oulès R, Barducci JP, Mourad G, Fourcade J, et al. *Mycobacterium haemophilum* and *Mycobacterium xenopi* associated infection in a renal transplant patient. Clin Nephrol. 1985;23:46–9.
5. Adékambi T, Reynaud-Gaubert M, Greub G, Gevaudan MJ, La Scola B, Raoult D, et al. Amoebal coculture of "*Mycobacterium massiliense*" sp. nov. from the sputum of a patient with hemoptoic pneumonia. J Clin Microbiol. 2004;42:5493–501.
6. Simmon KE, Pounder JI, Greene JN, Walsh F, Anderson CM, Cohen S, et al. Identification of an emerging pathogen, *Mycobacterium massiliense*, by *rpoB* sequencing of clinical isolates collected in the United States. J Clin Microbiol. 2007;45:1978–80.
7. Kim HY, Yun YJ, Park CG, Lee BH, Cho YK, Park BJ, et al. Outbreak of *Mycobacterium massiliense* infection associated with intramuscular injections. J Clin Microbiol. 2007;45:3127–30.
8. Viana-Niero C, Lima KV, Lopes ML, da Silva Rabello MC, Marsola LR, Brilhante VC, et al. Molecular characterization of *Mycobacterium massiliense* and *Mycobacterium bolletii* in outbreaks of infections after laparoscopic surgeries and cosmetic procedures. J Clin Microbiol. 2008;46:850–5.
9. Clinical and Laboratory Standards Institute (CLSI). Susceptibility testing of *Mycobacteria*, *Nocardia*, and other aerobic actinomycetes: approved standard M24-A. Wayne (PA): The Institute; 2003.
10. Brown-Elliott BA, Wallace RJ Jr. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. Clin Microbiol Rev. 2002;15:716–46.

Address for correspondence: Enrico Tortoli, Regional Reference Center for Mycobacteria, Microbiology Laboratory, Careggi Hospital, Viale Morgagni 85, 50134 Florence, Italy; email: enrico.tortoli@unifi.it

Bovine Kobuviruses from Cattle with Diarrhea

To the Editor: A new species of kobuvirus, named U-1 strain, was first recognized in 2003 as a cytopathic contaminant in a culture medium of HeLa cells that had been used for >30 years in the laboratory (*1*). The RNA genome of the U-1 strain comprises 8,374 nucleotides; the genome organization is analogous to that of picornaviruses. Morphologically, the U-1 strain resembles the Aichi virus, but genetically it is distinct (*1*). Therefore, the U-1 strain is classified as a new species of genus *Kobuvirus* in the family *Picornaviridae*, and it is called *bovine kobuvirus* (*1*). To date, the genus *Kobuvirus* consists of 2 species, *Aichi virus* and *bovine kobuvirus* (*2*). The Aichi virus is associated with acute gastroenteritis in humans (*3–5*); bovine kobuvirus infection has been detected only in cattle (*1*).

Only 1 report has described the discovery and epidemiologic features of bovine kobuvirus (*1*). Of serum samples from 72 healthy cattle, 43 (59.7%) were positive for neutralizing antibody against bovine kobuvirus U-1 standard strain at a titer of ≥16. In addition, 12 (16.7%) of 72 stool samples collected from the cattle were positive for the bovine kobuvirus genome by reverse transcription–PCR (RT-PCR) (*1*). This finding suggested that bovine kobuvirus is common and that the virus particles could be detected in the stool samples of infected cattle. We therefore conducted an epidemiologic survey of bovine kobuvirus and report detection of this virus in stool samples from calves with diarrhea during 2001–2004 in Chiang Mai Province, Thailand.

From November 2001 to July 2004, a total of 72 fecal specimens were collected. The age of the calves ranged from 7 to 49 days. The presence of bovine kobuvirus in fecal specimens was detected by using RT-PCR with a protocol modified from the method described by Yamashita et al. (*1*). All the bovine kobuvirus strains detected in our study were analyzed further by direct sequencing of their PCR amplicons with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an automated sequencer (ABI 3100, Applied Biosystems). The nucleotide sequences of these portions were compared with those of reference strains available in the GenBank database by using BLAST (*6*). Phylogenetic and molecular evolutionary analyses were conducted by using MEGA version 3.1 (*7*). The nucleotide sequences of bovine kobuvirus strains described in this study were deposited in GenBank under accession nos. EF659450–EF659455.

The bovine kobuvirus was detected by the RT-PCR screening method in 6 (8.3%) of the 72 fecal specimens collected. The partial 3D regions of all 6 bovine kobuviruses exhibited highly conserved sequences of 99.3%–100% nucleotide and 100% amino acid

Figure. Phylogenetic analysis of the partial nucleotide sequence encoding the 3D region of bovine kobuviruses isolated in this study and other reference strains recognized to date. The tree was generated on the basis of the neighbor-joining method using the MEGA 3.1 program (7). Scale bar indicates branch length for a 5% nucleotide difference.

identities to each other. In addition, by searching for the closest sequences in the databank, we found that these sequences were closely related to all 13 bovine kobuvirus reference strains available in the GenBank database (U-1, K-35, K-36, K-49, K-38, N-5, K-3, K-4, K-6, K-60, N-2, K-44, and K-55); nucleotide and amino acid sequence identities ranged from 91.0% to 95.0% and 97.4% to 99.4%, respectively. Phylogenetic analysis of partial 3D nucleotide sequences of our bovine kobuvirus strains, together with those of all published bovine kobuvirus reference strains available in the GenBank database, is shown in the Figure. The Aichi virus standard strain was included in the tree as an outlier virus. The phylogenetic tree confirmed that all 6 virus strains belonged to bovine kobuvirus and formed a tight cluster in a monophyletic branch with other published bovine kobuvirus reference strains, but they were distantly related to the Aichi virus standard strain.

Detection and characterization of bovine kobuvirus strains from different geographic areas are important for understanding the worldwide distribution, heterogeneity, and association of bovine kobuvirus with enteric disease in cattle. Our findings indicate the role of kobuviruses in diarrheal disease in cattle and provide additional information on their relationship to bovine kobuviruses reported previously.

This research was supported by the Core University System Exchange Program under the Japan Society for the Promotion of Science, coordinated by the Graduate School of Medicine, the University of Tokyo, Japan, and Mahidol University, Thailand. The study was also supported in part by the Research Fund from the Faculty of Medicine, Chiang Mai University, Thailand.

Pattara Khamrin,*† Niwat Maneekarn,‡ Supatra Peerakome,‡ Shoko Okitsu,† Masashi Mizuguchi,* and Hiroshi Ushijima*†

*University of Tokyo, Tokyo, Japan; †Aino Health Science Center, Tokyo; and ‡Chiang Mai University, Chiang Mai, Thailand

References

1. Yamashita T, Ito M, Kabashima Y, Tsuzuki H, Fujiura A, Sakae K. Isolation and characterization of a new species of kobuvirus associated with cattle. J Gen Virol. 2003;84:3069–77.
2. Racaniello VR. Picornaviridae: the viruses and their replication. In: Knipe DM and Howley PM, editors. Fields virology, vol. 1. 5th ed. Philadelphia: Lippincott Williams & Wilkins; 2006. p. 795–838.
3. Oh DY, Silva PA, Hauroeder B, Diedrich S, Cardoso DD, Schreier E. Molecular characterization of the first Aichi viruses isolated in Europe and in South America. Arch Virol. 2006;151:1199–206.
4. Pham NT, Khamrin P, Nguyen TA, Kanti DS, Phan TG, Okitsu S, et al. Isolation and molecular characterization of Aichi viruses from fecal specimens collected in Japan, Bangladesh, Thailand, and Vietnam. J Clin Microbiol. 2007;45:2287–8.
5. Yamashita T, Sakae K, Kobayashi S, Ishihara Y, Miyake T, Mubina A, et al. Isolation of cytopathic small round virus (Aichi virus) from Pakistani children and Japanese travelers from Southeast Asia. Microbiol Immunol. 1995;39:433–5.
6. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403–10.
7. Kumar S, Tamura K, Nei M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform. 2004;5:150–63.

Address for correspondence: Hiroshi Ushijima, Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan; email: ushijima-hiroshi@jcom.home.ne.jp

Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have one Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

4. Kapoor G, Aneja S. Nutritional disorders in adolescent girls. Ind Pediatr 1992;29:969–73.
5. Das DK, Biswas R. Nutritional status of adolescent girls in a rural area of North 24 Paraganas district, West Bengal. Ind J Pub Health 2005;49:18–21.
6. Das P, Ray SK, Joardar GK, *et al.* Nutritional profiles of adolescents in rural community of Hooghly district in West Bengal. Ind J Pub Health 2007;51:20–1.
7. Lohman TG, Roche AF, Martorell R. Anthropometric Standardization Reference Manual. Chicago: Human Kinetics Books, 1988.
8. World Health Organization. Measuring change in nutritional status. Geneva: World Health Organization, 1985.

Acknowledgements

Financial assistance of UGC, WBDST and ICMR is gratefully acknowledged. The authors express their thanks to the school authorities for their help and co-operation.

Correspondence: Kaushik Bose, Department of Anthropology, Vidyasagar University, Midnapore 721 102, West Bengal, India. E-mail <banda@vsnl.net>.

The Role of Flexible Bronchoscopy in Pediatric Pulmonary Tuberculosis

Tuberculosis (TB) disease in children is complicated by the unique challenges that it poses to the clinician. Despite the availability of effective preventive measures and chemotherapy, the prevalence of TB is increasing in the developing world and in much of the industrialized world as well, the incidence of TB in developed countries has declined over the last several decades. However, in recent years, there has been an increase in reported cases, especially in urban areas. In developing countries, the risk for TB infection and disease is relatively uniform in the population; annual rates of infection often exceed 2%. In pediatric patients, pulmonary tuberculosis (PTB) is a major TB infection, but the diagnosis of PTB presents a challenge. The symptoms of PTB infection are often absent or less specific in the affected pediatric population. Because children aged <8 years old lack sputum production, it is difficult to diagnose by sputum specimen. Children with PTB typically have closed caseous lesions with a relatively small number of mycobacteria. The large cavitary population of tubercle bacilli seen in adults is usually absent in children. This is compounded by the difficulty in collecting sputum in children as they swallow the expectorate coming from the lungs. Furthermore, at present, diagnostic tests are costly, slow and lacking in sensitivity. Flexible bronchoscopy (FB) is a newly available tool in the investigation of pediatric pulmonary disease. FB has been used mainly to evaluate unresolved chronic respirator symptoms, and in certain circumstances, to obtain cultures from the immune system of compromised hosts. Therefore, the aim of this retrospective survey is to evaluate the role of FB in the diagnosis of pediatric PTB.

In order to evaluate the value of FB for the diagnosis of children with suspected PTB, clinical records were retrospectively reviewed in 206 patients, who were diagnosed with PTB from January 2002 to December 2006 at the Beijing Children's Hospital. All 206 patients were examined by chest X-ray. Purified protein derivative tuberculin skin test (PPD) results were interpreted according to the recommendations of the American Thoracic Society. FB was performed on every patient according to clinical records in our survey. Bronchoalveolar lavage (BAL) was obtained to isolate Mycobacterium tuberculosis by FB. The mean age was 4.5 ± 2.7 years. The most frequent symptoms were cough (90%) and fever (85%) in all patients. The PPD results of 150/181 patients were 10 mm or more. Characteristic chest X-ray findings of PTB were 98/206 (47.6%). Thirty-three patients with endobronchial tuberculosis (EBTB) (94.3%) were diagnosed by FB. Bronchoscopic findings of 206 cases were divided into four types by our previous study. The number of indomucous membrane, bronchial lumen, caseating, and blending type was 36, 19, 6 and 145, respectively. FB is a useful tool to diagnose pediatric PTB. FB should be performed especially when chest X-rays are abnormal or when clinical signs suggest PTB.

HUI DING, YUYAN MA, XIAOCHUN RAO, AANXIA JIAO *and* XICHENG LIU

Beijing Children's Hospital, Affiliated to Capital Medical University, Beijing 100045, China

doi:10.1093/tropej/fmn056
Advance Access Published on 8 July 2008

Correspondence: Dr Hui Ding, Beijing Children's Hospital, Affiliated to Capital Medical University, 56 South Lishi Road, Beijing 100045, P.R. China. Tel.: +86 10 68020657; Fax: +86 10 68020657. Email <huidingtc@yahoo.com.cn>.

Epidemiological and Molecular Analysis of Astrovirus Gastroenteritis in Dhaka City, Bangladesh

Human astrovirus (HAstV) may be the second most common cause of viral gastroenteritis in young children, with rotavirus being the first [1]. HAstVs are members of the *Astroviridae* family. They are non-enveloped viruses possessing a single stranded RNA of positive polarity [2].

A total of 917 fecal specimens were collected from infants and children with acute gastroenteritis in Dhaka City, Bangladesh, during the period of October 2004 to September 2005. The fecal specimens were diluted with distilled water to 10% suspensions, and clarified by centrifugation at 10 000 g for 10 min. The viral genome was extracted from 140 μl of a 10% fecal suspension using a spin-column technique according to the instructions in the QIAamp® Viral RNA Mini Kit Handbook. Briefly, a pair of published primers (PreCAP1 and 82b) for amplifying capsid region of astrovirus was used [3]. The nucleotide sequences of polymerase chain reaction products positive for astrovirus were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc., CA, USA). Phylogenetic and molecular evolutionary analyses were conducted using the MEGA version 3.2 software package [4]. The following reference strains and accession numbers were used in the phylogenetic analysis: HAstV1 Oxf (L23513), HAstV2 (L13745), HAstV3 (L38505), HAstV4 (L38506), HAstV6 (L38507), HAstV7 (L38508), HAstV-8 (Z66541), PA-COD379-BR (DQ917390), Bcn1.3/97-00 (AF348755), Melb1F/97 (AF175254), JAPAS115 (AB000287), Melb1E/97 (AF175253), Ven835 (AF211956), Arg158 (AY324858), Dresden/04 (AY720892), 5333/JP (EU022556) and 5079/JP (EU022555).

All fecal specimens were tested for the presence of astrovirus by reverse transcription-polymerase chain reaction. The results shown in Table 1 revealed that astrovirus was detected in 4 out of 917 (0.4%) specimens tested. These findings suggested that HAstV is also a causative agent for acute gastroenteritis in infants and children in Dhaka City, Bangladesh. In the present study, astrovirus infection was found in December, January, June and September.

The age of astrovirus infection ranged from 3 to 11 months. It was found that the common clinical symptoms of astrovirus-infected patients were dehydration (100%), abdominal pain (75%) and fever (25%).

TABLE 1
Clinical features of the patients infected with astrovirus

Sign and symptoms	Number of infants and children	(%)
Dehydration	4/4	100
Vomiting	0/4	0
Abdominal pain	3/4	75
Fever	1/4	25
Hospitalization	4/4	100
3–5 times loose stools within 24 h	1/4	25
≥6 times loose stools within 24 h	3/4	75

All the HAstV amplicons were characterized for serotypes and genetic relationship with the reference strains based on their capsid regions. Their partial nucleotide sequences were compared to each other as well as to reference HAstV strains available in the DDBJ DNA/GenBank database by BLAST. This study showed that all Bangladeshi astrovirus sequences belonged only to serotype I (Fig. 1). HAstV detected in this study was closely homologous at the nucleotide and amino acid level.

The nucleotide sequence data of all Bangladeshi HAstV had been submitted to the GenBank (NCBI, Bethesda, MD) and had been assigned accession number EU333900–EU333903, respectively.

This is the first time that data on nucleotide analysis of Bangladeshi astrovirus were reported. Nucleotide sequence analysis indicated that the common serotype I strains were circulating among Bangladeshi children and infants.

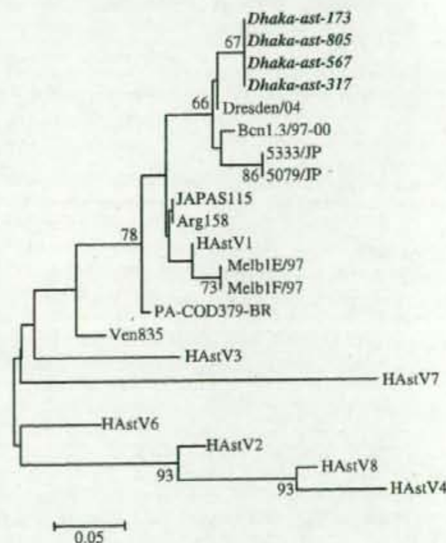

FIG. 1. Phylogenetic tree of the 300 bp region of ORF2 of 4 HAstVs-1 from Dhaka City. The tree was constructed from partial amino acid sequences of capsid region of astrovirus. Reference strains of astrovirus were selected from DDBJ/GenBank under the accession number indicated. Bangladeshi astrovirus was highlighted in italic. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. The Genbank accession numbers of this study are: EU333900–EU333903, respectively.

In conclusion, to our knowledge this is the first 1 year molecular epidemiological research of HAstV in Bangladesh.

SHUVRA KANTI DEY,[a] ABUL ISLAM,[b]
MASASHI MIZUGUCHI,[a] SHOKO OKITSU,[a,c] *and*
HIROSHI USHIJIMA[a,c]

[a]*Department of Developmental Medical Sciences*
Institute of International Health
Graduate School of Medicine
The University of Tokyo, Tokyo, Japan
[b]*Department of Microbiology Dhaka University*
Dhaka-1000, Bangladesh, and
[c]*Aino Health Research Center*
Aino University, Tokyo, Japan

doi:10.1093/tropej/fmn057
Advance Access Published on 9 July 2008

References

1. Guix S, Caballero S, Villena C, *et al.* Molecular epidemiology of astrovirus infection in Barcelona, Spain. J Clin Microbiol 2002;40:133–9.
2. Clark B, McKendrick M. A review of viral gastroenteritis. Curr Opin Infect Dis 2003;241–6.
3. Yan H, Yagyu F, Okitsu S, *et al.* Detection of norovirus (GI,GII), sapovirus and astrovirus in fecal samples using reverse transcription single-round multiplex PCR. J Virol Methods 2003;14:37–44.
4. Kumar S, Tamura K, jakobsen IB, *et al.* MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 2002;17:1244–5.

Acknowledgement

This study was supported by Grants-in-aid from the Ministry of Education and the Ministry of Health, Labor and Welfare, Japan.

Correspondence: Hiroshi Ushijima.
E-mail <ushijima-hiroshi@jcom.home.ne.jp>.

Virus Genes (2008) 37:314–321
DOI 10.1007/s11262-008-0274-5

Genetic characterization of group C rotavirus isolated from a child hospitalized with acute gastroenteritis in Chiang Mai, Thailand

P. Khamrin · S. Peerakome · R. Malasao ·
M. Mizuguchi · S. Okitsu · H. Ushijima ·
N. Maneekarn

Received: 4 June 2008 / Accepted: 31 July 2008 / Published online: 12 August 2008
© Springer Science+Business Media, LLC 2008

Abstract During an epidemiological survey of human rotavirus infection in Chiang Mai, Thailand, from 2002 to 2004, in which 263 stool specimens tested, one isolate of group C rotavirus was detected from a two-year-old child admitted to hospital with acute gastroenteritis. The human group C rotavirus, named CMH004/03, was characterized further by molecular analyses of its VP4, VP6, and VP7 gene segments as well as determination of RNA pattern by polyacrylamide gel electrophoresis (PAGE). Molecular characterization of VP4, VP6, and VP7 genes by sequence analyses showed high levels of sequence identities with those of human group C rotavirus reference strains isolated worldwide at 95.2% to 99.4% on nucleotide and 97.5% to 100% on amino acid levels. In contrast, the CMH004/03 strain exhibited far lesser nucleotide and amino acid sequence identities at 67.7% to 84.1% and 68.7% to 91.3%, respectively, when compared with those of porcine and bovine group C rotaviruses. Phylogenetic analyses of VP4, VP6, and VP7 genes clearly confirmed that the CMH004/03 strain clustered in a monophyletic branch with other human group C rotavirus reference strains and distantly related to the clusters of animal group C rotavirus strains. In addition, the RNA electrophoretic migration pattern of CMH004/03 showed a typical pattern (4-3-2-2) of group C rotavirus. To our knowledge, this study is the second report of group C rotavirus infection in pediatric patients in Thailand after it was reported for the first time about two decades ago.

Keywords Rotavirus · Group C · VP4 · VP6 · VP7 · Thailand

P. Khamrin · S. Okitsu · H. Ushijima
Aino Health Science Center, Aino University, Tokyo, Japan

P. Khamrin · M. Mizuguchi · H. Ushijima
Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

S. Peerakome · N. Maneekarn (✉)
Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand
e-mail: nmaneeka@mail.med.cmu.ac.th

R. Malasao
School of Medical Sciences, Naresuan University Phayao, Phayao, Thailand

Introduction

Rotavirus is the major cause of acute gastroenteritis in infants, young children, and a large variety of young animal species. The virus is a member of the family *Reoviridae* with a double stranded RNA genome of 11 segments, surrounded by a triple-layered capsid protein [1]. The outermost viral capsid primarily comprises of two proteins, VP4 and VP7. The middle layer is formed exclusively by the most abundant protein of the virus, VP6, which defined group and subgroup (SG) specificities. The innermost layer is formed by the VP2 protein, a known RNA binding protein and the transcription enzymes VP1 (viral RNA polymerase) and VP3 (guanylyltransferase) are attached as a heterodimeric complex to the inside of the VP2 innermost surface protein [1]. On the basis of antigenic cross-reactivity and genomic properties of VP6, thus far, rotavirus is classified into seven antigenically distinct groups, A to G. All seven groups have been found to associate with infection in various animal species, but only groups A, B, and C are associated with diarrhea in humans [1–3]. Group A rotavirus is the major cause of childhood

diarrhea worldwide, and accounts for approximately 454,000-705,000 deaths annually [4]. Human group B rotavirus was first described in China as the cause of nationwide epidemics of diarrhea in adults [5]. Group C rotavirus was first detected from piglets in 1980 [6] and later confirmed as a human pathogen by Bridger et al. [2]. Since then, group C rotavirus has been detected from both sporadic episodes and outbreaks of gastroenteritis throughout the world [7–12]. Host range restriction of group C rotavirus has been documented; only human, porcine, canine, and bovine species were found to be infected with group C rotaviruses [2, 6, 13–15]. All human group C rotaviruses analyzed so far have shown a high degree of sequence conservations, and several evolutionary studies also indicated that human group C rotavirus may have evolved from the same ancestor and possibly belonged to a single genotype [12, 16–18].

In Thailand, only one report of group C rotavirus infection in patients with diarrhea had been documented in 1989 [19]. Since then, it has never been reported as a cause of acute gastroenteritis in Thailand. In contrast to group C rotavirus, which is a less common viral agent causing diarrhea, group A rotavirus is reported as the most predominant viral agent causing diarrhea in humans worldwide [4, 20]. The accumulated data of group A rotavirus surveillance studies in Thailand indicated that group A rotavirus is the leading pathogen that causes diarrhea in children, and it is responsible for about 27% to 58% of diarrhea in hospitalized cases [21–24]. In this study, the group C rotavirus strain was detected from a child hospitalized with acute gastroenteritis. Its VP4, VP6, and VP7 genes were characterized further by nucleotide sequence analyses and the genetic relationship with other group C rotavirus strains circulating worldwide was also investigated.

Materials and methods

Specimen collection

A total of 263 fecal specimens were collected from pediatric patients aged less than 5 years, who were hospitalized with diarrhea at McCormick Hospital, Chiang Mai, Thailand. The study period was from January 2002 to December 2004. Group A rotavirus was first screened in the fecal specimens collected [24]. In addition, the incidence of group C rotavirus infection was also examined by using the same set of fecal samples, with the aim of having an overview of molecular epidemiology of human group C rotavirus in children hospitalized with acute gastroenteritis in Chiang Mai, Thailand.

Detection of group C rotavirus

The RNA genome of rotavirus was first extracted from 10% fecal suspension supernatant using the QIAamp viral RNA Mini Kit (Qiagen, Germany). The presence of group C rotavirus in fecal specimens was detected by RT-PCR using the protocol described previously [25]. A forward primer, G8NS1 (nt 353 to 374) 5′-ATTATGCTCAGACTATCGC-CAC-3′, was used in combination with the reverse primer G8NA2 (nt 683 to 704) 5′-GTTTCTGTACTAGCTGGT-GAAC-3′, for the amplification of a partial VP7 gene of the group C rotavirus, which specifically generated a PCR amplicon of 352 bp. The group C rotavirus isolated in this study was analyzed further by nucleotide sequence analysis of its VP4, VP6, and VP7 genes.

Polyacrylamide gel electrophoresis of rotavirus RNA

The RNA electrophoretic pattern of group C rotavirus (CMH004/03) was determined based on the method described previously [26]. The dsRNA segments were separated on 10% (w/v) polyacrylamide gel, and the migration pattern was visualized by silver staining [27].

Amplification of the VP4, VP6, and VP7 genes

The partial VP4 gene of group C rotavirus (CMH004/03) was reverse transcribed and amplified using the consensus primers GrC VP4-1F (nt 1 to 20) 5′-GGCTTAAAAAGTAG AGATCG-3′ and GrC VP4-1243R (nt 1224 to 1243) 5′-CC AGGATATGATCCTA-3′ [18]. The full length of the VP6 gene was amplified using T777 (nt 1 to 21) 5′-GGCTTTAAA AATCTCATTCAC-3′ and T778 (nt 1334 to 1353) 5′-AGCC ACATAGTTCACATTTC-3′ primer pairs. For amplification of the full-length VP7 gene, consensus primers T348 (nt 1 to 22) 5′-GGCTTTAAAAAAGAAGAAGCTG-3′ and T349 (nt 1042 to 1063) 5′-AGCCACATGATCTTGTTTAC GC-3′ were used [28].

Sequence and phylogenetic analyses of the VP4, VP6, and VP7 genes

The PCR amplicons of the entire VP6 and VP7 genes as well as the partial sequence of the VP4 gene were gel purified with a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI), and sequenced in both directions using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an automated sequencer (ABI 3100; Applied Biosystems, Foster City, CA). The primers used for amplification of the VP4, VP6, and VP7 genes were also used as sequencing primers. The nucleotide and deduced amino acid sequences were compared with those of group C rotavirus reference strains

available in the NCBI (National Center for Biotechnology Information) GenBank database using the BLAST (Basic Local Alignment Search Tool) server [29]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 [30].

Nucleotide sequence accession numbers

The nucleotide sequences of the group C rotavirus strain (CMH004/03) described in this study were deposited in the GenBank database. The accession numbers for the VP4, VP6, and VP7 sequences are EF641109, EF641110, and EF641111, respectively.

Results

Detection of the human group C rotavirus

From a total of 263 fecal specimens included in this study, 98 (37.3%) were positive for group A rotavirus [24], while one isolate (0.4%), named CMH004/03, was identified as group C rotavirus by RT-PCR using primers specific for amplification of VP7 gene of group C rotavirus. The rotavirus strain CMH004/03 was collected in January 2003 from a 2-year-old child admitted with acute gastroenteritis at McCormick Hospital, Chiang Mai province, Thailand. At the time of admission, the patient had fever, nausea, and vomiting without abdominal pain, and had mild dehydration with watery diarrhea for up to 3 to 4 times per day.

Electropherotype of group C rotavirus strain

The RNA electrophoretic migration patterns of the group C rotavirus strain CMH004/03 was characterized by PAGE and compared with RNA patterns of group A rotavirus reference strains available in our laboratory. The CMH004/03 strain exhibited a characteristic of 4-3-2-2 migration pattern, which was typical for group C rotavirus, while group A rotavirus showed a 4-2-3-2 RNA migration pattern (Fig. 1).

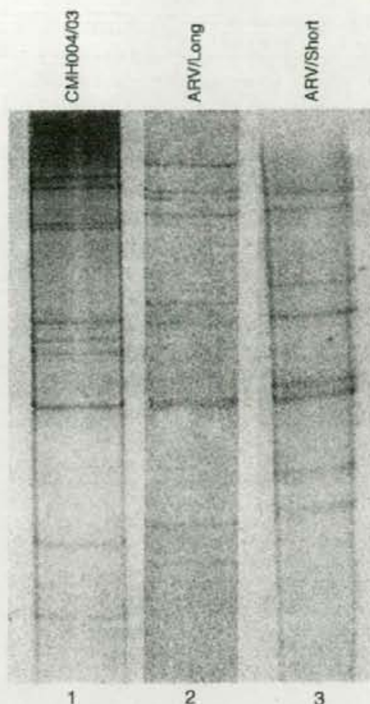

Fig. 1 Electrophoretic migration patterns of rotavirus genomic RNAs. Lane 1 is group C rotavirus detected in this study (CMH004/03), while lanes 2 and 3 are group A rotaviruses representing long and short patterns. The viral RNA were analyzed by electrophoresis in a 10% polyacrylamide gel and visualized by silver nitrate staining

Analysis of the VP7 gene sequence

The VP7 gene of the group C rotavirus strain CMH004/03 was 1,063 nucleotides long, which encoded a polypeptide of 332 amino acid residues, similar to other human group C rotaviruses. Comparative analysis of the nucleotide and deduced amino acid sequences of the full-length VP7 gene with those of human and animal group C rotavirus reference sequences indicated that the VP7 gene of CMH004/03 was closely related to those of other human group C rotavirus reference strains (95.2% to 98.8% on nucleotide and 97.5% to 99.3% on amino acid sequence levels) (Table 1). In addition, the VP7 gene of CMH004/03 showed the highest level of nucleotide sequence identity with Japanese group C rotavirus strain OT-99 at 98.8%, whereas the amino acid sequence was most closely related to that of the Columbian group C rotavirus strain Javeriana at 99.3% identity. In contrast, CMH004/03 exhibited sequences distantly related to the porcine and bovine group C rotaviruses at 74.3% to 84.1% nucleotide and 71.0% to 87.0% amino acid sequence identities. A phylogenetic analysis of the VP7 nucleotide sequences of the CMH004/03 strain provided a molecular basis for its genetic relatedness to other human group C rotavirus strains and formed the closest genetic lineage with human group C rotavirus strains Javeriana, OT-99, and KW408 (Fig. 2).

Analysis of the VP4 gene sequence

The partial VP4 nucleotide sequence (nucleotide 1 to 1,241) and deduced amino acid sequence (407 amino acid residues) of CMH004/03 were determined and compared

Table 1 Comparison of nucleotide and amino acid sequence identities of the VP7 gene segment of the CMH004/03 strain with those of the representative group C rotavirus strains

Strain	Host species	Country	Accession No.	Sequence identity (%)	
				Nucleotide	Amino acid
KA4/1018	Human	Sweden	AF225563	97.8	98.4
Uppsala/1004	Human	Sweden	AF225560	98.1	98.7
T97/330	Human	Sweden	AF225558	98.0	98.7
KW290	Human	Japan	AB086967	97.9	97.5
Solano	Human	Argentina	AF120478	97.6	96.9
KU166	Human	Japan	AB086966	97.5	98.1
S-1	Human	Japan	U20995	97.9	97.8
E93	Human	Japan	U20992	98.4	98.7
Javeriana	Human	Columbia	AJ549087	98.6	**99.3**
OT-99	Human	Japan	AB086969	**98.8**	99.0
KW408	Human	Japan	AB086968	98.7	98.7
ASP/87	Human	Australia	U20990	97.8	98.1
OK118	Human	Japan	D87543	97.8	97.5
88-220	Human	Japan	M61100	97.4	98.1
A90L	Human	Australia	AY392446	97.2	98.1
Bristol	Human	UK	X77257	97.4	98.1
Preston	Human	UK	X77258	97.2	98.1
BCN21	Human	Spain	AM118023	97.3	97.8
T97/167	Human	Sweden	AF225553	97.5	98.1
Santiago	Human	Chile	U20996	97.8	98.4
Belem	Human	Brazil	X77256	96.9	97.8
ad1015	Human	USA	U20994	97.8	98.4
ad957	Human	USA	U20993	97.8	98.4
Fuan	Human	China	U20987	97.3	98.1
Moduganari	Human	Africa	AF323979	96.3	98.4
V460	Human	India	AY803724	95.3	97.8
Jajeri	Human	Africa	AF323982	95.2	97.8
Cowden	Porcine	USA	M61101	84.1	87.0
WH	Porcine	USA	U31749	82.9	84.6
HF	Porcine	USA	U31748	75.4	71.0
Yamagata	Bovine	Japan	AB108681	74.3	73.7
Shintoku	Bovine	Japan	U31750	75.3	74.0

Bold characters indicate the highest percentage of sequence identity

with those of established VP4 group C rotavirus reference strains available in the GenBank database. The sequence comparison shown in Table 2 indicates that the partial VP4 gene of CMH004/03 was closely related to those of other human group C rotavirus reference strains at 95.5% to 98.3% on nucleotide and 97.5% to 99.5% on amino acid sequence levels. The highest sequence identity was found with the human rotavirus reference strain 208 detected in China at 98.3% and 99.5% of nucleotide and amino acid sequence levels, respectively. The strains detected in animals exhibited much less nucleotide and amino acid sequence identities with the CMH004/03 strain, ranging from 67.7% to 70.4% and 68.7% to 69.5%, respectively. A phylogenetic tree constructed from the nucleotide sequence of CMH004/03 and those of group C rotavirus reference sequences from both human and non-human origins is shown in Fig. 3. It was again found that the VP4 gene sequence of the Thai group C rotavirus strain formed exclusively in a monophyletic branch with other human group C rotavirus reference strains, and appeared to be most closely related to the Chinese 208 strain. Moreover, based on the VP4 sequences, group C rotavirus strains could be divided into three discrete lineages, one comprised only human rotavirus strains, whereas the other two distinct lineages were formed by porcine and bovine group C rotavirus strains, respectively.

Analysis of the VP6 gene sequence

Analysis of the complete VP6 gene sequence of CMH004/03 revealed that the VP6 gene of this strain was 1,353 nucleotides long, which encoded a polypeptide of 395

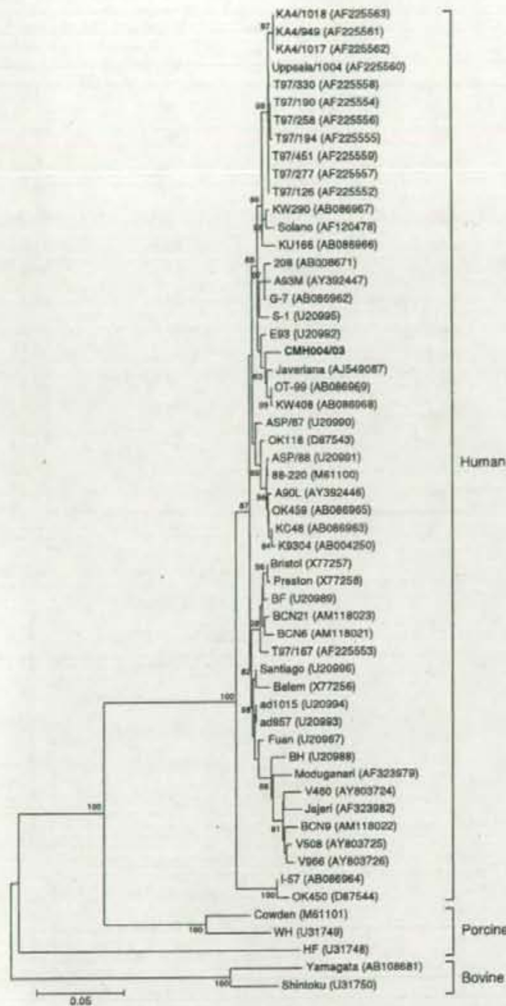

Fig. 2 Phylogenetic analysis of the nucleotide sequences derived from the VP7 genes of the human group C rotavirus strain CMH004/03 and other group C rotavirus reference strains. The tree was constructed based on the neighbor-joining method using the MEGA 3.1 program. The number presented in the branch nodes is the bootstrap value and the scale bar indicates the branch lengths for 5% nucleotide difference

amino acid residues, like other human group C rotavirus strains. Overall comparison of the VP6 nucleotide and deduced amino acid sequences of CMH004/03 with those of the existing group C rotavirus reference strains revealed a high level of sequence identity with human group C rotaviruses circulating all over the world, ranging from 97.2% to 99.4% on nucleotide and 99.2% to 100% on amino acid levels (Table 3). The VP6 sequence of CMH004/03 was most closely related to the Chinese Wu82 strain at 99.4% nucleotide and 100% amino acid identities. However, the CMH004/03 exhibited far lesser sequence identity to the strains detected in animals (81.6% to 82.9% on nucleotide and 87.5% to 91.3% on amino acid sequence levels). A phylogenetic analysis of the VP6 nucleotide sequences confirmed the finding that the CMH004/03 strain clustered with other human group C rotavirus reference strains in a monophyletic branch and formed the same lineage with the Wu82 strain detected previously in China (Fig. 4). In addition, all human group C rotavirus strains analyzed were distantly related to group C rotaviruses of porcine and bovine origins.

Discussion

From the literature search, the only initial report of group C rotavirus in Thailand was carried out in the central area in 1989 [19]. In that study, four cases were identified as group C rotavirus infections by PAGE, but no genetic information of those group C rotaviruses were described in that report. Since then, the human group C rotavirus has not been reported as a cause of acute gastroenteritis in Thailand until our report in the present study. The intensive epidemiological surveillances of rotaviruses have been conducted consecutively in Chiang Mai, Thailand, since 1984 [21, 26]. Most of those studies focused only on group A rotavirus infections, which is the main causative agent of gastroenteritis that accounted for approximately 17% to 58% of hospitalized children in Chiang Mai area [21, 23, 24], whereas group C rotavirus infections among hospitalized infants and young children have never been investigated in this area before. The present study reports the detection of human group C rotavirus strain (CMH004/03) from a sporadic case of a young girl admitted to hospital with acute gastroenteritis in 2003 in Chiang Mai area, northern Thailand. This is the first report describing the molecular genetic characteristics of group C rotavirus in the country. The low rate of detection (0.4%) in the studied populations suggests that group C rotavirus is an uncommon cause of acute gastroenteritis in hospitalized patients. This observation is in line with a number of publications of group C rotaviruses in sporadic cases of diarrhea with a very low prevalence rate, for instance, 1.2% in Japan [31], 2.3% in Bangladesh [18], 1.8% in Nigeria [28], 1% in Argentina [32], and 0.6% in Hungary [33]. However, several investigators suggested that the patients infected with group C rotaviruses were older (more than 5 years of age) than those infected with human group A rotaviruses [19, 31, 33]. It is possible that very low incidence of group C rotavirus observed in the present study may be because the patients being enrolled in the study were less than

Table 2 Comparison of nucleotide and amino acid sequence identities of the VP4 gene segment of the CMH004/03 strain with those of the representative group C rotavirus strains

Strain	Host species	Country	Accession No.	Sequence identity (%)	
				Nucleotide	Amino acid
V966	Human	India	AY795896	96.9	98.2
V460	Human	India	AY795897	96.7	98.5
BCN9	Human	Spain	AM118025	97.0	98.0
V508	Human	India	AY795895	96.7	98.0
Moduganari	Human	Africa	AF323980	96.9	98.0
Jajeri	Human	Africa	AF323981	96.9	98.0
208	Human	China	AB008670	**98.3**	**99.5**
A93 M	Human	Australia	AY395070	97.0	97.5
A87 J	Human	Australia	AY395069	96.5	98.5
Belem	Human	Brazil	X79441	96.2	98.2
Bristol	Human	UK	X79442	96.3	97.7
BCN21	Human	Spain	AM118026	95.5	97.7
BCN6	Human	Spain	AM118024	95.5	97.5
Cowden	Porcine	USA	M74218	70.4	69.5
Shintoku	Bovine	Japan	U26551	67.7	68.7

Bold characters indicate the highest percentage of sequence identity

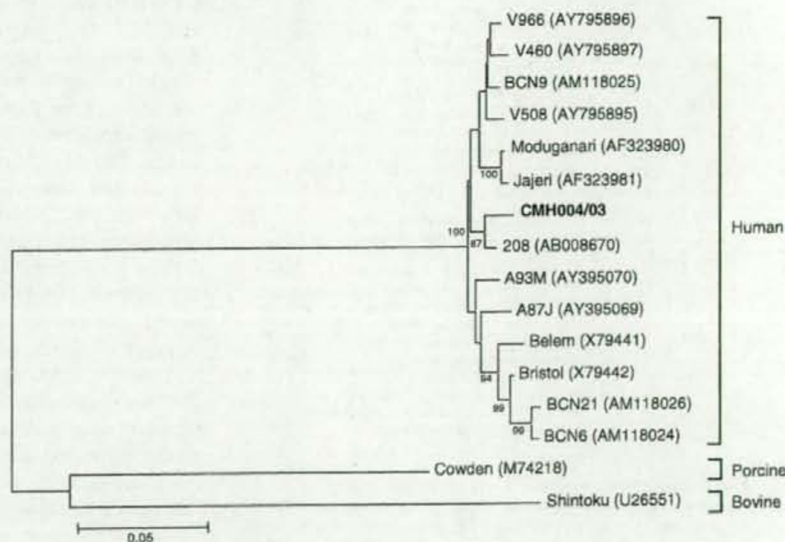

Fig. 3 Phylogenetic analysis of the nucleotide sequences derived from partial VP4 genes of the human group C rotavirus strain CMH004/03 and other group C rotavirus reference strains. The tree was constructed based on the neighbor-joining method using the MEGA 3.1 program. The number presented in the branch nodes is the bootstrap value and the scale bar indicates the branch lengths for 5% nucleotide difference

5 years old. In addition, Abid et al. reported that 31% of fecal specimens collected in Spain contained the inhibitors of a RT-PCR assay [34]. Since our detection method for group C rotaviruses was RT-PCR, the possibility that a very low detection rate of group C rotaviruses observed in the present study might be due to inhibitors containing in the stool specimens could not be ruled out.

The nucleotide and deduced amino acid sequences of gene encoding for three major structural proteins, VP4, VP6, and VP7, of human group C rotavirus isolated from different regions of the world displayed remarkable sequence conservations, and greater differences have been noted between human and animal group C rotavirus strains [17, 28, 35]. Up to now, a formal classification system of group C rotaviruses has not yet been well established. Based on nucleotide and deduced amino acid sequence analyses, however, at least four G (VP7), three P (VP4), and three VP6 genetic clusters have been proposed [12, 14, 18]. Phylogenetic analyses of the VP4, VP6, and VP7 genes of our CMH004/03 strain are in good agreement with those of the previous studies [12, 14, 18]. Furthermore, phylogenetic analysis also confirmed that the genome

Table 3 Comparison of nucleotide and amino acid sequence identities of the VP6 gene segment of the CMH004/03 strain with those of the representative group C rotavirus strains

Strain	Host species	Country	Accession No.	Sequence identity (%)	
				Nucleotide	Amino acid
V508	Human	India	AY795898	98.2	99.4
V966	Human	India	AY786571	98.3	99.7
BCN9	Human	Spain	AM118019	98.2	99.7
V460	Human	India	AY786570	98.3	99.7
Jajeri	Human	Africa	AF325805	98.0	98.9
208	Human	China	AB008672	98.3	98.7
Wu82	Human	China	EF528570	**99.4**	**100.0**
BCN6	Human	Spain	AM118018	97.6	99.2
BCN21	Human	Spain	AM118020	97.7	99.4
Bristol	Human	UK	X59843	98.1	99.7
Preston	Human	UK	M94156	98.0	99.7
Moduganari	Human	Africa	AF325806	97.2	99.7
Belem	Human	Brazil	M94155	97.4	99.7
WD534tc	Bovine	USA	AF162434	81.9	87.5
Cowden	Porcine	USA	M94157	82.9	91.3
Shintoku	Bovine	Japan	M88768	81.6	88.1
Yamagata	Bovine	Japan	AB108680	81.7	88.6

Bold characters indicate the highest percentage of sequence identity

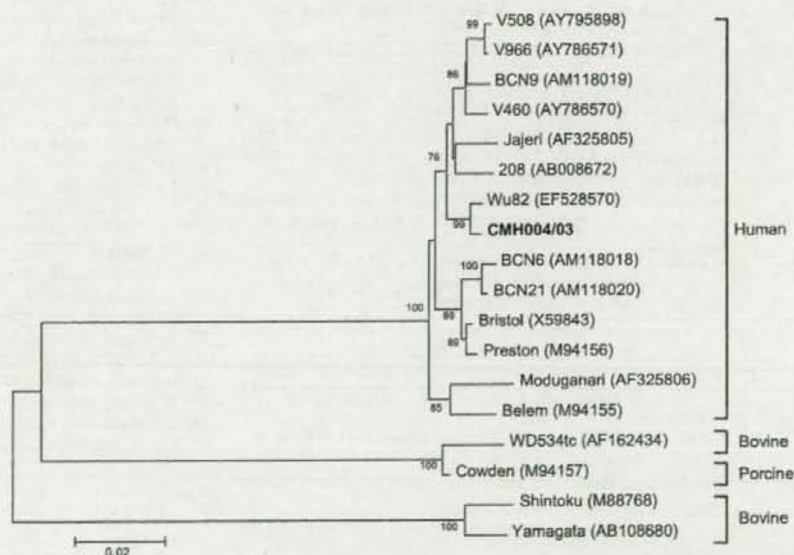

Fig. 4 Phylogenetic analysis of the nucleotide sequences derived from the VP6 genes of the human group C rotavirus strain CMH004/03 and other group C rotavirus reference strains. The tree was constructed based on the neighbor-joining method using the MEGA 3.1 program. The number presented in the branch nodes is the bootstrap value and the scale bar indicates the branch lengths for 2% nucleotide difference

sequences of VP4, VP6, and VP7 genes formed a monophyletic branch with other human group C rotavirus reference strains. In addition, sequence analyses of the VP4, VP6, and VP7 genes revealed that the sequences are highly conserved (over 95% on nucleotide and 97% on amino acid levels) and the host range is restricted. It is noteworthy to point out that all the genes of our human group C rotavirus strain CMH004/03 were most closely related to those of human group C rotavirus reference strains reported previously in Asian countries, including Japan (OT-99, KW408) and China (208 and Wu82), than those of other regions of the world, with the exception of one isolate (Javeriana) from Columbia.

In conclusion, the present study reports the first genetic evolutionary analysis of human group C rotavirus detected in Thailand by analyzing of the VP4, VP6, and VP7 genes.

The results demonstrated a close genetic relationship between the VP4, VP6, and VP7 genes of our human group C rotavirus strain CMH004/03 and those of other human group C rotavirus reference strains circulating in other parts of the world. The findings imply that human group C rotaviruses detected worldwide, so far, may have evolved from a common ancestor.

Acknowledgments This research was supported by Grant-in-Aid for Scientific Research under the JSPS Postdoctoral Fellowships and by Grants-in-Aid from the Ministry of Education and Sciences and the Ministry of Health, Labor and Welfare, Japan. In addition, the study was also supported in part by the Research Fund from the Faculty of Medicine, Chiang Mai University, Thailand.

References

1. M.K. Estes, in *Fields virology*, 4th edn., ed. by D.M. Knipe, P.M. Howley, D.E. Griffin (Lippincott, Williams and Wilkins, Philadelphia, PA, 2001), pp. 1747–1785
2. J.C. Bridger, S. Pedley, M.A. McCrae, J. Clin. Microbiol. **23**, 760 (1986)
3. Z.Y. Fang, Q. Ye, M.S. Ho, H. Dong, S. Qing, M.E. Penaranda et al., J. Infect. Dis. **160**, 948 (1989)
4. U.D. Parashar, C.J. Gibson, J.S. Bresse, R.I. Glass, Emerg. Infect. Dis. **12**, 304 (2006)
5. L.J. Saif, B. Jiang, Curr. Top. Microbiol. Immunol. **185**, 339 (1994)
6. L.J. Saif, E.H. Bohl, K.W. Theil, R.F. Cross, J.A. House, J. Clin. Microbiol. **12**, 105 (1980)
7. N.A. Cunliffe, W. Dove, B. Jiang, B.D. Thinwda Cert, R.L. Broadhead, M.E. Molyneux et al., Pediatr. Infect. Dis. J. **20**, 1088 (2001). doi:10.1097/00006454-200111000-00018
8. T.G. Phan, S. Nishimura, M. Okame, T.A. Nguyen, P. Khamrin, S. Okitsu et al., J. Med. Virol. **74**, 173 (2004). doi:10.1002/jmv.20162
9. R.D. Schnagl, K. Boniface, P. Cardwell, D. McCarthy, C. Ondracek, B. Coulson et al., J. Clin. Microbiol. **42**, 2127 (2004). doi:10.1128/JCM.42.5.2127-2133.2004
10. M. Kuzuya, M. Hamano, M. Nishijima, R. Fujii, H. Ogura, M. Tanaka et al., Jpn. J. Infect. Dis. **58**, 255 (2005)
11. A. Steyer, M. Poljsak-Prijatelj, T. Bufon, M. Sedmak, L. Vidmar, J.Z. Mijovski et al., J. Med. Virol. **78**, 1250 (2006). doi:10.1002/jmv.20687
12. I. Abid, S. Guix, M. Aouni, R. Pinto, A. Bosch, J. Clin. Virol. **38**, 78 (2007). doi:10.1016/j.jcv.2006.09.012
13. E.H. Bohl, L.J. Saif, K.W. Theil, A.G. Agnes, R.F. Cross, J. Clin. Microbiol. **15**, 312 (1982)
14. B. Jiang, J.R. Gentsch, H. Tsunemitsu, L.J. Saif, R.I. Glass, Virus Genes **19**, 85 (1999). doi:10.1023/A:1008196824879
15. P. Otto, P. Schulze, W. Herbst, Arch. Virol. **144**, 2467 (1999). doi:10.1007/s007050050659
16. A.S. Grice, P.R. Lambden, E.O. Caul, I.N. Clarke, J. Med. Virol. **44**, 166 (1994). doi:10.1002/jmv.1890440209
17. B. Jiang, H. Tsunemitsu, P.H. Dennehy, I. Oishi, D. Brown, R.D. Schnagl et al., Arch. Virol. **141**, 381 (1996). doi:10.1007/BF01718407
18. M. Rahman, S. Banik, A.S. Faruque, K. Taniguchi, D.A. Sack, M. Van Ranst et al., J. Clin. Microbiol. **43**, 4460 (2005). doi:10.1128/JCM.43.9.4460-4465.2005
19. M.E. Penaranda, W.D. Cubitt, P. Sinarachatanant, D.N. Taylor, S. Likanonsakul, L. Saif et al., J. Infect. Dis. **160**, 392 (1989)
20. N. Santos, Y. Hoshino, Rev. Med. Virol. **15**, 29 (2005). doi:10.1002/rmv.448
21. N. Maneekarn, H. Ushijima, Pediatr. Int. **42**, 415 (2000). doi:10.1046/j.1442-200x.2000.01251.x
22. C. Jiraphongsa, J.S. Bresee, Y. Pongsuwanna, P. Kluabwang, U. Poonawagul, P. Arporntip et al., J. Infect. Dis. **192**, 87 (2005). doi:10.1086/431508
23. P. Khamrin, S. Peerakome, L. Wongsawasdi, S. Tonusin, P. Sornchai, V. Maneerat et al., J. Med. Virol. **78**, 273 (2006). doi:10.1002/jmv.20536
24. P. Khamrin, S. Peerakome, S. Tonusin, R. Malasao, S. Okitsu, M. Mizuguchi et al., J. Med. Virol. **79**, 1775 (2007). doi:10.1002/jmv.20960
25. H. Yan, T.A. Nguyen, T.G. Phan, S. Okitsu, Y. Li, H. Ushijima, Kansenshogaku Zasshi **78**, 699 (2004)
26. A. Hasegawa, A. Mukoyama, H. Suzuki, S. Inouye, S. Chearskul, P. Thongkrajai et al., J. Diarrhoeal Dis. Res. **5**, 165 (1987)
27. A.J. Herring, N.F. Inglis, C.K. Ojeh, D.R. Snodgrass, J.D. Menzies, J. Clin. Microbiol. **16**, 473 (1982)
28. M.I. Adah, A. Wade, M. Oseto, M. Kuzuya, K. Taniguchi, J. Med. Virol. **66**, 269 (2002). doi:10.1002/jmv.2141
29. S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, J. Mol. Biol. **215**, 403 (1990)
30. S. Kumar, K. Tamura, M. Nei, Brief. Bioinform. **5**, 150 (2004). doi:10.1093/bib/5.2.150
31. M. Kuzuya, R. Fujii, M. Hamano, M. Nishijima, H. Ogura, J. Med. Virol. **79**, 1219 (2007). doi:10.1002/jmv.20910
32. A.A. Castello, M.H. Argüelles, G.A. Villegas, A. Olthoff, G. Glikmann, J. Med. Virol. **67**, 106 (2002). doi:10.1002/jmv.2198
33. K. Bányai, B. Jiang, A. Bogdán, B. Horváth, F. Jakab, E. Meleg et al., J. Clin. Virol. **37**, 317 (2006). doi:10.1016/j.jcv.2006.08.017
34. I. Abid, S. Guix, M. Aouni, R. Pintó, A. Bosch, J. Clin. Virol. **38**, 78 (2006). doi:10.1016/j.jcv.2006.09.012
35. H. Tsunemitsu, B. Jiang, L.J. Saif, Arch. Virol. **141**, 705 (1996). doi:10.1007/BF01718328

Journal of Medical Virology 80:1749–1755 (2008)

Genetic Diversity of Norovirus, Sapovirus, and Astrovirus Isolated From Children Hospitalized With Acute Gastroenteritis in Chiang Mai, Thailand

Rungnapa Malasao,[1] Niwat Maneekarn,[1] Pattara Khamrin,[2] Chansom Pantip,[1] Supin Tonusin,[3] Hiroshi Ushijima,[2] and Supatra Peerakome[1*]

[1]*Faculty of Medicine, Department of Microbiology, Chiang Mai University, Chiang Mai, Thailand*
[2]*Aino Health Science Center, Aino University, Tokyo, Japan*
[3]*Department of Pediatrics, McCormick Hospital, Chiang Mai, Thailand*

Norovirus (NV), sapovirus (SV), and human astrovirus (HAstV) are important causes of acute gastroenteritis in infants and young children. This study investigated the prevalence of NV, SV, and HAstV infections in children hospitalized with acute gastroenteritis in Chiang Mai, Thailand from May 2000 to March 2002. Fecal specimens were tested for NV, SV, and HAstV by reverse transcription polymerase chain reaction (RT-PCR) using degenerate specific primers. These viruses were characterized further by sequence and phylogenetic analyses of the partial capsid gene. From 296 fecal specimens tested, 13.5% (40 of 296) were positive for NV, SV, and HAstV. Of these, NV most predominant, with a prevalence of 60% (24 of 40), of which 17.5% were NVGI and 42.5% were NVGII. Of note, one specimen was positive for both NVGI and SV. SV was detected in 25%, while HAstV was detected in 17.5%. Analysis of nucleotide and amino acid sequences revealed that NVGI strains comprised GI/3, GI/4, GI/6, GI/7, and GI/13 genotypes. Among NVGII strains, approximately half of them belonged to genotype GII/4 (Lordsdale virus cluster), followed by GII/3, GII/10, GII/1, GII/6, GII/8, and GII/15. Analysis of SV sequences revealed that SVGI (Manchester virus) was more common than SVGII (London virus). The SV genotypes detected in this study belonged to SVGI/1, SVGI/4, SVGI/5, SVGII/1, and SVGII/2, whereas the HAstV belonged to genotypes HAstV-1, HAstV-2, HAstV-3, and HAstV-5. The findings suggest that NV, SV, and HAstV are important enteric viruses cocirculating among hospitalized children in Chiang Mai, Thailand. ***J. Med. Virol. 80:1749–1755, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: noroviruses; sapoviruses; astroviruses; acute gastroenteritis; Chiang Mai; Thailand

© 2008 WILEY-LISS, INC.

INTRODUCTION

Acute gastroenteritis is one of the most common diseases in infants, children, and adults worldwide. During the first 5 years of life, every child will contract diarrheal disease, which enhances the risk of dehydration and nutritional deficiency [Jiraphongsa et al., 2005]. In addition to rotavirus (RV), human caliciviruses and astrovirus have emerged as etiologic causes of acute gastroenteritis in this age group. Norovirus (NV) and sapovirus (SV) are classified into the family *Caliciviridae* and are members of nonenveloped, positive-sense, single-stranded RNA viruses. Their genome contain approximately 7,300–8,300 nucleotides long, and a genome-linked protein (VPg) at the 5′ terminus and a poly A tail at the 3′ terminus [Bertolotti-Ciarlet et al., 2003]. Human astrovirus (HAstV) is a member of the family *Astroviridae*, and has a small (28–30 nm in diameter), round, nonenveloped characteristic. The genome is a positive-sense, single-stranded RNA of approximately 6,800 nucleotides in length [Schnagl et al., 2002].

NVs can be divided into five distinct genogroups based on the variation in the capsid gene sequences, in which strains belonging to GI, GII, and GIV are found in humans, whereas GIII and GV are found in cows and mice, respectively [Zheng et al., 2006]. Recently, NVs were classified into 8, 17, 1, 1, and 1 genotypes in GI, GII, GIII, GIV, and GV, respectively [Zheng et al., 2006]. SVs are divided into five genogroups (GI to GV) based on the

Grant sponsor: Faculty of Medicine; Grant sponsor: Graduate School of Chiang Mai University, Chiang Mai, Thailand.

*Correspondence to: Supatra Peerakome, Faculty of Medicine, Department of Microbiology, Chiang Mai University, Chiang Mai, Thailand. E-mail: speerako@mail.med.cmu.ac.th

Accepted 7 May 2008

DOI 10.1002/jmv.21244

Published online in Wiley InterScience (www.interscience.wiley.com)

difference of capsid gene sequences, in which GI, GII, GIV, and GV are found in humans, while GIII is found in porcine species [Farkas et al., 2004]. Recently, Akihara et al. [2005] reported that SV strains are classified further into 16 genetic clusters/genotypes (8 in GI, 5 in GII, 1 each in GIII, GIV, and GV) based on the differences of partial capsid amino acid sequences. HAstVs can be divided into eight distinct serotypes or genotypes based on the antigenic difference or variation in the capsid gene sequences: serotype/genotype 1 to 8, in which a high concordance between results of serotyping and genotyping was observed [Sakamoto et al., 2000].

In Thailand, epidemiological studies of NV, SV, and HAstV are less frequent than those of RV. The study conducted in Chiang Mai in 2000 and 2001 [Hansman et al., 2004] reported that NV infection in children hospitalized with diarrhea was 7.6%, while SV infection was 3.8%. One specimen (0.95%) was an NV/SV mixed infection. Later, during 2002 and 2003, Guntapong et al. [2004] reported 11 NV and 9 SV single infections, and 3 were NV/SV mixed infections from a total of 80 stool specimens collected from children hospitalized with acute gastroenteritis in 5 different geographical areas of Thailand (Sa Kaeo, Chanthaburi, Songkhla, Nong Khai, and Tak). For HAstV, the frequency of detection rate ranged from 8.6% to 14% in hospitalized children [Herrmann et al., 1991; Echeverria et al., 1994]. In 2004, a report from Bangkok demonstrated that HAstV was the cause of a neonatal gastroenteritis outbreak, which occurred in the nursery of a maternity ward at Ramathibodi Hospital. HAstV was detected in 4 of 13 (30.7%) diarrheic neonates and 1 member of the nursery staff who had diarrhea [Sirinavin et al., 2006].

This study reports the prevalence and molecular epidemiology of norovirus, sapovirus, and astrovirus infections in sporadic gastroenteritis among hospitalized children in Chiang Mai, Thailand from May 2000 to March 2002.

MATERIALS AND METHODS

Specimen Collection

Two hundred ninety-six fecal specimens were collected from children hospitalized with diarrhea in four different hospitals and one private clinic in Chiang Mai province between May 2000 and March 2002. The ages of the subjects ranged from neonate up to 5 years old.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Viral RNA was extracted from 10% fecal supernatant using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany). For RT, the viral RNA was reverse transcribed according to the manufacturer's instruction (Fermentas, Lithuania Glen Burnie, MD). The presence of NV (GI and GII), SV, and HAstV was detected by RT-PCR using the protocol described previously [Yan et al., 2003]. A forward primer, G1-SKF (nt 5,342–5,361) 5′-CTGCC-CGAATTYGTAAATGA-3′, was used in combination with the reverse primer, G1-SKR (nt 5,653–5,671) 5′-CCAACCCARCCATTRTACA-3′, for the amplification of NVGI, which specifically generated a PCR amplicon of 330 bp. For NVGII identification, a forward primer, COG2F (nt 5,003–5,028) 5′-CARGARBC-NATGTT-YAGRTGGATGAG-3′, was used in combination with the reverse primer, G2-SKR (nt 5,367–5,389) 5′-CCR-CCNGCATRHCCRTTRTACAT-3′, which generated a PCR product size of 387 bp. For SV detection, a 434 bp fragment was generated using the forward primer, SLV5317 (nt 5,083–5,105) 5′-CTCGCCACCTA-CRAW-GCTTGGTT-3′, and reverse primer, SLV5749 (nt 5,516–5,494) 5′-CGGRCYTCAAAVSTACCBCCC-CA-3′. For amplification of HAstV, a forward primer, PreCAP1 (nt 4,235–4,255) 5′-GGACTGCAAAGCAG-CTTCGTG-3′, was used in combination with the reverse primer, 82b (nt 4,934–4,953) 5′-GTGAGCCACCAGC-CATCCCT-3′, which generated a PCR product size of 719 bp. All of the positive samples were analyzed further for their genotypes by nucleotide sequence and phylogenetic analyses.

Sequence and Phylogenetic Analyses

The PCR products were purified by the QIAquick Gel Extraction Kit (Qiagen) and sequenced by using the BigDye Terminator Cycle Sequencing Kit (Perkin Elmer-Applied Biosystems, Inc., Foster City, CA) on an automated DNA sequencer (ABI 3100; Perkin Elmer-Applied Biosystems, Inc.). The primers employed for amplification of the partial capsid genes were also used as sequencing primers. The nucleotide sequences obtained were translated into amino acid sequences using the GeneDoc program, and compared to those of NV (GI and GII), SV, and HAstV reference strains available in the GenBank using the BLAST program. The genotypes of NV (GI and GII) and SV were classified based on the recent capsid region classification scheme of Zheng et al. [2006] and Akihara et al. [2005], respectively. The serotypes/genotypes of HAstV were assigned in comparison with those of the reference strains by analysis of their amino acid sequences.

Nucleotide Sequence Accession Numbers

The partial nucleotide sequences of the capsid gene were deposited in GenBank under the accession number EU363852–EU363875 for NV strains, EU363876–EU363885 for SV strains, and EU363886–EU363892 for HAstV strains. The following capsid gene sequences of reference strains published in the GenBank were used in the phylogenetic analysis: NVGI: Boxer/01/US (AF538679), Chiba/00/JP (AB042808), DSV395 (U04469), Hesse (AF093797), Musgrove/89/UK (AJ277614), Norwalk/68 (M87661), Saitama T36GI/01/JP (AB112133), Southampton (L07418),