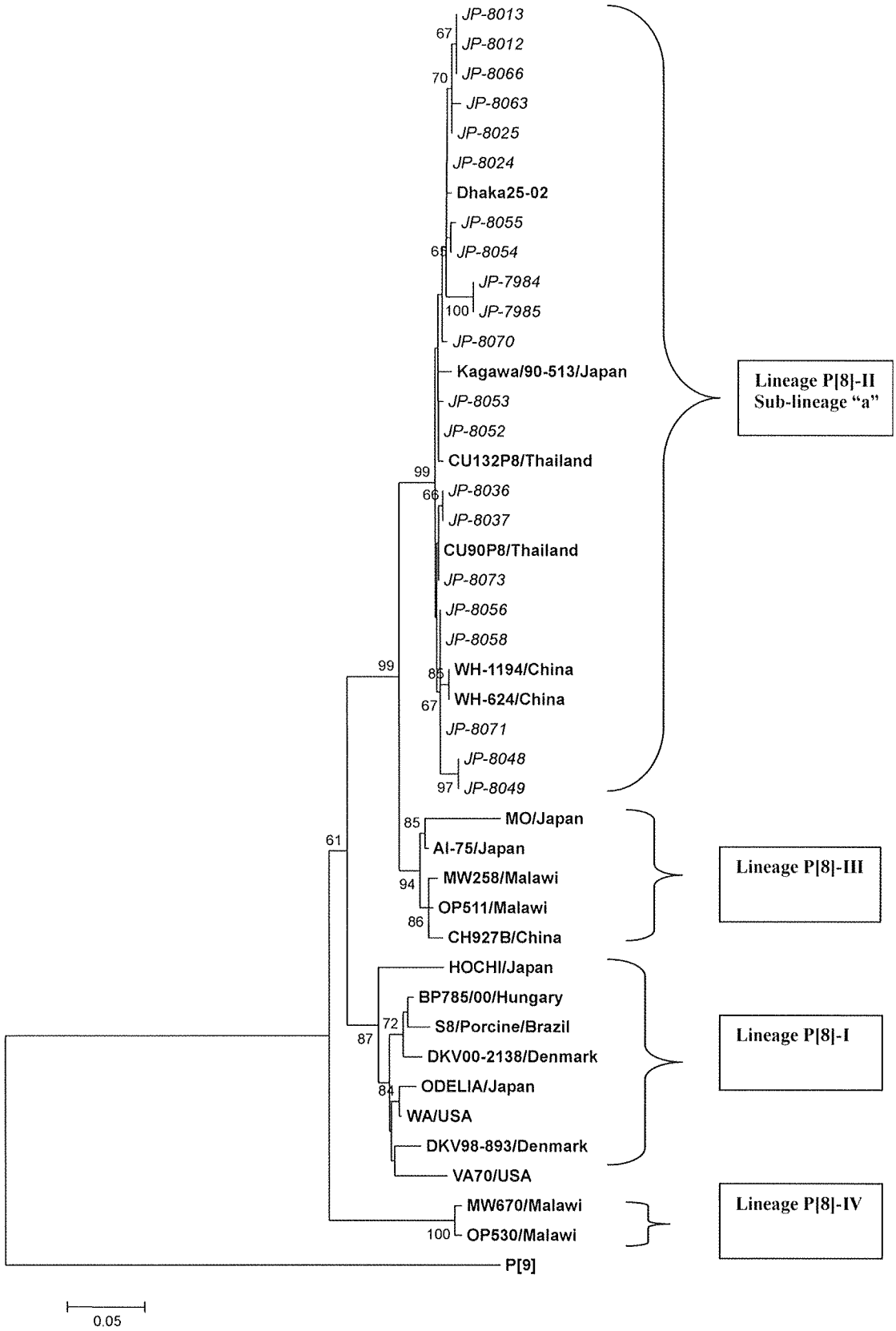

Fig. 2. Phylogenetic tree of nucleotide sequence of Japanese rotavirus P[8] isolates. The tree was constructed from nucleotide sequences of VP4 of rotavirus P[8] isolates detected in Japan in 2006–2007. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. Reference strains of rotavirus P[8] were selected from DNA Data Bank of Japan (DDBJ/GenBank) under the accession number indicated in bold. Japanese P[8] strains in 2006–2007 were highlighted in italic.

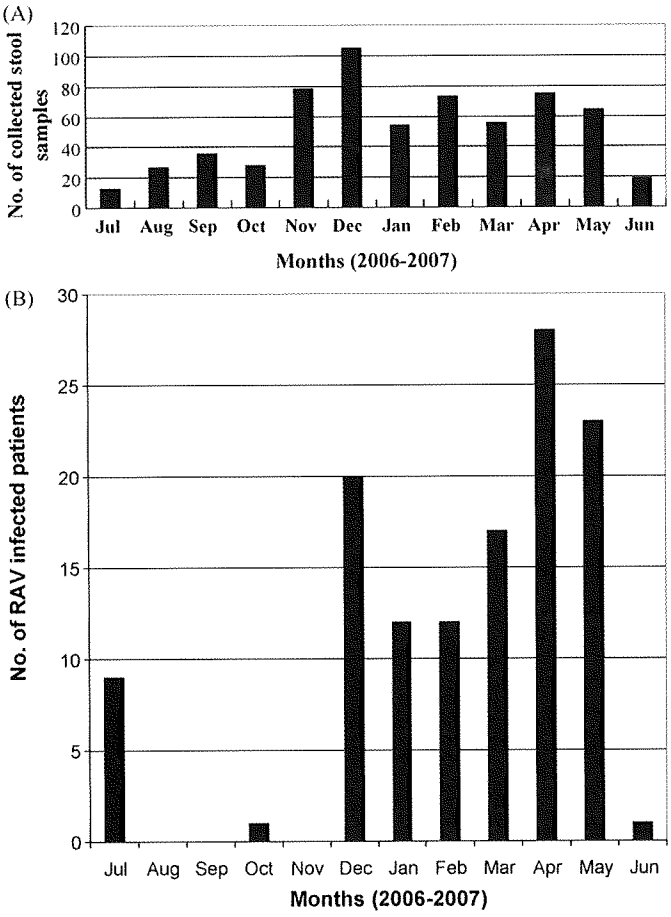

Fig. 3. (A) Number of stool samples collected each month from infants and children with acute gastroenteritis in Japan during July 2006 to June 2007. (B) Seasonal pattern of rotavirus gastroenteritis among infants and children with acute gastroenteritis in Japan during July 2006 to June 2007.

probably caused by other etiological agents including but not limited to norovirus, sapovirus, astrovirus and bacteria.

The present study describes the epidemiology of rotavirus infection among children and infants in five prefectures (cities) of Japan between 2006 and 2007. RAV was detected continuously between December and June (Fig. 3B) with peak prevalence from March to May ($P < 0.002$). Prevalence between August and November ranged from 0% to 5.2%. While previous reports have described rotavirus infections during the colder months of the year, some studies have failed to detect this seasonal correlation. The findings of this study are in agreement with studies conducted by Phan et al. (2007a) and Zhou et al. (2003) demonstrating that the peak of rotavirus infection in Japan is in March and April.

One of the main goals of this study was to characterize the VP7 (G genotype) and VP4 (P genotype) gene segments of the Japanese rotavirus strains. We identified most of the globally common rotavirus types (G1, G2, G3, and G9) in our study. G1 was identified with a very high prevalence (58.5%) and followed by G9 (20.3%), G2 (11.4%), and G3 (7.3%). Extensive molecular epidemiological studies globally have indicated that G1 is the predominant serotype in circulation (Nguyen et al., 2007; Phan et al., 2007a; Yan et al., 2005; Zhou et al., 2003; Maneekarn and Ushijima, 2000). In Japan, serotype G1P[8] strains were predominant in the same areas during the 2004/2005 and 2005/2006 rotavirus seasons at levels of 46.4% and 70.1%, respectively (Phan et al., 2007a,b). While G1 strains appear to circulate at relatively constant levels, G3 strains have decreased in number since 2004, causing 32.9% cases in 2004/2005, 17.9% cases in 2005/2006 and 7.3% cases in 2006/2007 (Yoshinaga et al., 2006; Phan et al., 2007a,b).

In Japan, a G9 strain was first detected in Tokyo in the 1996/1997 rotavirus season (Okame et al., 2006). Between 1998 and 2003, G9 strains persisted in the Japanese community and were detected at levels of 15.3% (Yoshinaga et al., 2006; Okame et al., 2006). Since 2004, serotype G9 strains have been increasing in number and while they were not detected in the 2004/2005 season in Japan, these strains were detected in 6.8% cases in 2005/2006 and in this study were the second most common genotype in 20.3% rotavirus infections.

Analysis of the P-types from Japanese specimens collected during the 2006/2007 season revealed P[8] (84.5%; 104/123) and P[4] (13.8%; 17/123). This result was consistent with the previous findings on rotavirus epidemiology in Japan and the region of South-east Asia (Nguyen et al., 2007; Phan et al., 2007a; Yan et al., 2005; Zhou et al., 2003; Maneekarn and Ushijima, 2000). Between 2003 and 2006, the most common rotavirus genotype was P[8] (76%) and non-P[8] strains constituted around 20%. In addition, two P[6] strains were also detected in this study. While the P[6] genotype is more common in rotavirus infections in Africa (Santos and Hoshino, 2005), these strains have detected in China (Fang et al., 2002). Recently, five rare porcine P[6] strains have been detected in Vietnam (Nguyen et al., 2007). To our knowledge, this is the first report of P[6] RAV in human infections in Japan.

The most predominant genotype detected in the study was G1P[8] in 58.5% cases, followed by G9P[8] (18.7%). In fact, the four most common strains globally including G1P[8], G2P[4], G3P[8] and G4P[8] accounted for 77.2% (95/123) cases. This result was consistent with other previous studies in other parts of Asia (Dey et al., 2009a,b; Phan et al., 2007a,b; Nguyen et al., 2007; Maneekarn and Ushijima, 2000). This result supports the fact that second generation rotavirus vaccine (Rotarix, Rotateq) may be effective for reducing rotavirus infection in Japan.

Japanese G1 RV clustered into two genetic lineages, lineage I with 24% (5 of 21) and lineage II with 76% (16 of 21). Within lineage II, all 16 strains belonged to sublineage IIa Within lineage I, 3 RAV G1 strains, belonged to sublineage Ia which had great identities at the nucleotide as well as amino acid level. On the other hand, other 2 strains belonged to sublineage Ic. RAV P[8] sequences in this study were classified into one distinct lineage, lineage II and sublineage IIa. P[8] strains analyzed in this study belonged to Asian cluster and strains which had great identities at the nucleotide as well as amino acid level each other (96% to 99%).

The group A rotavirus has been associated with gastroenteritis in infants and children under 2 years of age.

It is clear from this study that RAV is still the cause of diseases in Japan. Future studies are needed in which systematic surveillance of gastroenteritis is done for prolonged time period and with harmonized methods in order to find explanations for the apparent emergence of rotavirus variants in populations.

Acknowledgements

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H3N2 Influenza A Virus Replicates in Immortalized Human First Trimester Trophoblast Cell Lines and Induces Their Rapid Apoptosis

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Keywords
Apoptosis, first trimester trophoblast, influenza A virus

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Problem
Epidemiological data suggested that pandemic influenza increased the risks of spontaneous abortion and premature labor, while seasonal influenza also increased the risk of schizophrenia in adolescence. However, their pathogenesis is so far unknown.

Method of study
The first trimester trophoblast cell lines, namely, Swan71 and HTR8 cells were challenged with A/Udorn/72 influenza virus (H3N2). At indicated time points, cells were examined for expression of influenza proteins. Viral replication in culture media, apoptosis and the expression of human leukocyte antigen (HLA)-G were also examined.

Results
Intracellular localization of viral proteins was observed. Twenty-four hours after inoculation, virus was detected in culture media while most cells fell into apoptosis. During apoptosis, expression of HLA-G was unchanged.

Conclusion
We revealed replication of low pathogenic influenza virus in the first trimester trophoblast cell lines. Placental damages are likely to be induced by direct cytopathic effects of influenza virus and subsequent apoptosis rather than down regulation of HLA-G expression and subsequent rejection by maternal immune system.

Introduction

Influenza is a highly contagious, acute viral febrile respiratory infection that results in global morbidity and mortality. During pregnancy poor outcome have been reported following the previous influenza pandemics.[1] During the influenza pandemic of 1918, remarkably high rates of spontaneous abortion and preterm birth were reported.[2,3] Asian influenza pandemic during 1957–58 also increased the rates of central nervous system defects and several other adverse outcomes, including birth defects, spontaneous pregnancy loss, fetal death, and preterm delivery,[4–6] while seasonal influenza did not increase the rates of pregnancy complications.[7] Recent studies have suggested the association of maternal influenza infection during pregnancy with three to seven-fold increase of schizophrenia in the offspring.[8–10] Brown et al. reported a

statistically significant association noted between elevated concentrations of cytokines or antibodies to influenza antigens in maternal serum and the incidence of schizophrenia in the offspring. They speculated that 4–21% of schizophrenia cases would have been prevented if the maternal infection had not occurred. Experimental rodent models have suggested that maternal influenza infection can influence the physiology, behavior, and neuropathology of adult offspring.[11] As viral RNA has not been detected in the fetal brain of influenza infected animals, these changes are speculated to be secondary to the maternal inflammatory responses, rather than results of direct viral effects.[12] To the best of our knowledge, the susceptibility and kinetics of influenza viruses in the early gestational trophoblasts have not been studied, while replication of influenza virus in freshly prepared cells from the term fetal membranes have been studied extensively by Uchide et al.[13]

In this study, we have hypothesized that invasive trophoblasts may be targets of influenza virus *in vivo* and *in vitro*. Taking into account the difficulty to obtain first-trimester human trophoblast primary cultures, we utilized the HTR8/SVneo and Swan71 (Sw.71) cell lines, both were derived from human first trimester extravillous trophoblast (EVT)[14] and have been employed as models of EVT cell proliferation, migration and invasiveness *in vitro*.[15–18] In this study, we observed that both cell lines were susceptible to non-pandemic influenza A (H3N2) virus and fell into apoptosis without remarkable reduction of human leukocyte antigen (HLA)-G expression.

Materials and methods

Cell Lines

The human first trimester trophoblast cell lines Swan71 (Sw.71, derived by telomerase-mediated transformation of a 7-week cytotrophoblast isolate described by Straszewski-Chavez)[19,20] and HTR8 (H8, originally were obtained from human first-trimester placenta and immortalized by transfection with a cDNA construct that encodes the simian virus 40 large T antigen)[14] were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sw.71) or RPMI 1640 (H8) normal growth medium (Gibco-Invitrogen, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 0.1 mM minimal essential medium non-essential amino acids, 1 mM sodium pyruvate, 100 nM of penicillin and streptomycin (Gibco-Invitrogen). Cells were cultured in monolayer at 37°C and 5% CO_2.

Virus

The influenza virus A/Udorn/307/72 (H3N2) maintained in Nihon University School of Medicine was propagated either in 10-day-old embryonic chicken eggs or in Madin-Darby canine kidney (MDCK) cells. MDCK cells were maintained in Eagle's minimum essential medium (EMEM) containing 10% FBS. Viral titers were checked with plaque formation assay before inoculation.

Viral Infections

Cells cultured in 6-well plates (2×10^5/well) were washed with phosphate-buffered saline (PBS) and infected with the influenza A/Udorn/72 at a multiplicity of infection of five (MOI 5) for 40 min at 37°C. After a 40-min adsorption, the inocula were removed, and cells were incubated with RPMI (for H8 cells) or DMEM (for Sw.71 cells) for the indicated times. Non-infectious influenza A/Udorn/72 was generated by incubating the virus at 56°C for 30 min, and inactivation was confirmed by the lack of cytopathic effect or replication on MDCK cell. For apoptosis examination, positive control was performed by incubating the cells with Actinomycin D (Nakarai Tesque, Inc., Kyoto, Japan), 1 μL of stock solution (1 mg/mL) per 10^5 cells.

Immunofluorescence Assay

H8 and Sw.71 cells grown on glass cover slips in 6-well plates were infected with the A/Udorn/72 at an MOI of 5. At the indicated time after infection, the cells were fixed with 4% paraformaldehyde solution for 10 min, washed with PBS, and incubated with rabbit anti-Udorn serum (1:1000 dilution), which had been prepared by immunizing rabbits with the purified virions as described previously,[21] for 1 hr at room temperature. Negative controls were placed by mock-virus treatment, heat-inactivated virus treatment as well as staining with rabbit sera without virus immunization. After washing with PBS, the cells were incubated with Alexa 488-conjugated anti-rabbit IgG solution, prepared using the Alexa Fluor 488 Protein Labeling Kit (Molecular Probes, Invitrogen, Tokyo, Japan), for 40 min at room temperature. After washing, the cells were mounted with Fluoromount G (SounthernBiotech, Birmingham, AL, USA), and the

fluorescent images were collected using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan).[22] Each experiment was repeated at least three times, and Fig. 1 shows typical influenza viral protein staining.

Hemagglutination Assay

Serial twofold dilutions of specimens were made in 50 μL of PBS on 96-well U-bottom plates. To each well, 50 μL of 0.5% chicken erythrocytes in PBS was then added. The plates were kept at 4°C for 1 hr, then the hemagglutination (HA) patterns were read and HA titers were determined from the last dilution showing complete HA. Each experiment was repeated three times and the average values of the three independent measurements are shown.

RNA Isolation, cDNA Synthesis and Real Time Polymerase Chain Reaction

Cultured trophoblasts in 6-well plates and supernatant were solubilized with 1mL of TRIZOL® (Invitrogen). RNA was first extracted with chloroform, precipitated with absolute ethanol. Next, after washing with 75% ethanol, RNA was re-dissolved in RNAse-free water. The quantity and quality of the RNA were determined by ultraviolet absorbance at 260 nm. Then, DNase digestion step was performed using DNase I (TaKaRa Bio Inc., Shiga, Japan).

For cDNA synthesis, reverse transcription (RT) was performed using PrimeScript™RT Reagent Kit (Perfect Real Time) (TaKaRa Bio Inc.) according to the manufacturer's protocol, in which, the primer T7-Uni12 was used for influenza examination.

Real-time polymerase chain reaction (PCR) was conducted with the SYBR Premix Ex Taq (Perfect Real Time) (TaKaRa Bio Inc.) according to the manufacturer's protocol with appropriate annealing temperature. For quantification of viral RNA copy numbers, dilutions of an external standard corresponding to 10^6 copies down to 1 copy of an influenza viral RNA solution were subjected to PCR in parallel using the primer pair T7 and vPB2qR3.[23] All

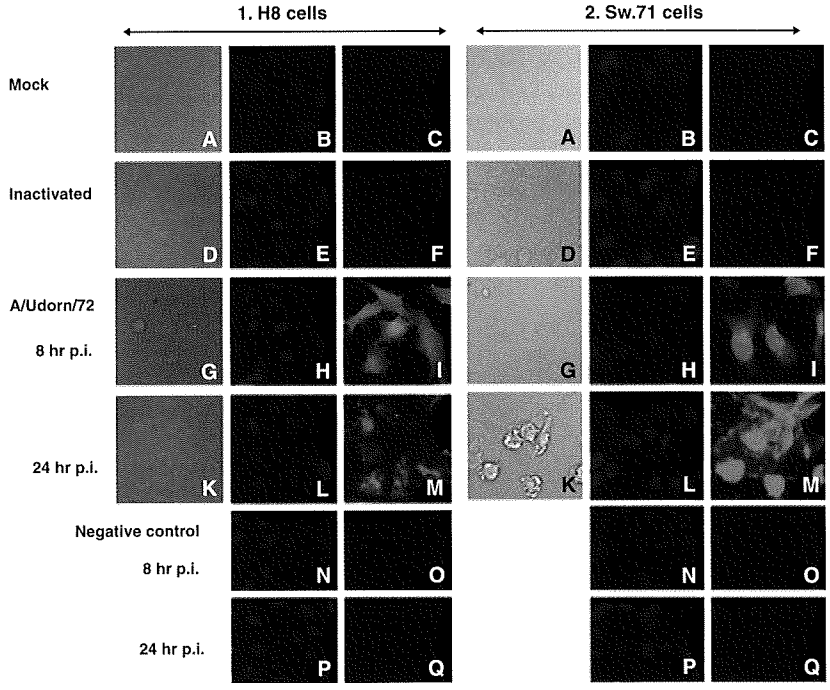

Fig. 1 Immunofluorescent images of H8 and Sw.71 cells infected with the A/Udorn/72 at an MOI of 5. At 8 h p.i. (panels 1G–I and 2G–I) and 24 h p.i. (panels 1K–M and 2K–M), the cells were fixed and stained for viral proteins using immunofluorescence with rabbit anti-Udorn serum. (1G, 1K, 2G, and 2K) differential interference contrast images; (1H, 1L, 2H, and 2L) the nuclei were counterstained with Hoesch stain (blue); (1I, 1M, 2I, and 2M) immunofluorescence staining of influenza viral proteins. Immunofluorescence (right) and corresponding brightfield (left) images are presented of the cells stained with antibodies for the viral proteins. Mock-infected cells and the cells incubated with the inactivated virus were also examined at 8 and 24 h p.i (panels 1A–F and 2A–F). (Panels 1N–Q and 2N–Q), Udorn-infected H8 and Sw.71 cells stained with rabbit serum as negative controls.

reactions were performed on the ABI Prism™ 7500 sequence detection system (Perklin-Elmer Applied Biosystems, Tokyo, Japan). The primers were obtained from TaKaRa Co.Ltd (Tokyo, Japan). Oligonucleotide sequences of the primers used in this study are shown in Table I.

Detection of Apoptosis

The early apoptosis was measured using Apo-Strand™ELISA apoptosis detection kit (BIOMOL, Plymouth Meeting, PA, USA).This detection system employs monoclonal antibody to single-stranded DNA (ssDNA) which occurs in apoptotic cells but not in necrotic cells or in cells with DNA breaks in the absence of apoptosis.[24] Tests were performed in cells grown in 96-well plates. For influenza virus inoculation Sw.71 and H8 cells were seeded (5000 cells/well) and cultured overnight before treatment. Cells were incubated with an MOI of 5 of influenza A/Udorn/72 and incubated for 18 hr. In brief, cells were fixed for 30 min with fixative indicated by the manufacturer and dried by incubating at 56°C for 20 min. Formamide was then added to the cells, and they were heated at 56°C for 30 min to denature DNA in apoptotic cells. Blocking solution was then added to all wells to block non-specific binding sites. Next, cells were incubated with antibody mixture for 30 min and rinsed with 1X wash buffer. After washing, cells were incubated with 100 μL of peroxidase substrate for 45 min, and absorbance was read using an ELISA plate reader at 405 nm. Reaction (color development) was stopped by the addition of 100 μL of 1% sodium dodecyl sulfate. Negative controls without viral inoculation, heat inactivated viruses and positive control with actinomycin D treatment were placed.

Flow Cytometric Analysis of HLA-G

For determination of the surface expression of HLA-G molecules, the cells (2×10^5) were washed twice with cold PBS containing 3% bovine serum albumin and then stained with FITC-conjugated mouse anti-human HLA-G monoclonal antibody (Clone MEM-G/11) (Exbio, Praha, Czech Republic) for 30 min at 4°C. After centrifugation, the cells were suspended in 0.5 mL of 1% paraformaldehyde and then subjected to flow cytometry analysis. Gated events were collected using the FACScalibur cytometer and analyzed with CellQuest software (Becton-Dickinson Biosciences, Tokyo, Japan).

Statistical Analysis

Analysis of variance was used for statistical analysis of the results. The resulting P-value <0.05 using Fisher's exact test was considered statistically significant.

Results

Imunofluorescence Detection of Influenza Virus Derived Antigens in H8 and Sw.71 Cells

H8 and Sw.71 cell lines were inoculated with non-pandemic influenza A Udorn/72 virus (H3N2). Intracellular localization of the virus in the infected cells was examined using immunofluorescence staining of the cells at indicated time points of 8 and 24 hr post infection (h p.i.) using rabbit anti-Udorn serum targeting with viral proteins of hemagglutinin protein (HA), nucleoprotein (NP)and matrix protein (M1). Non-immunofluorescence was detected in the mock-infected or heat-inactivated virus-treated cell (Fig. 1, panels 1A–F, and 2A–F) while strong intracellular localization of the virus was detected in the both cell lines at 8 and 24 h p.i. (Fig. 1, panels 1I, 1M for H8 cells and 2I, 2M for Sw.71 cells). Negative controls employing rabbit sera showed no immunofluorescence (Fig. 1, panels 1N–Q and 2N–Q). In addition, as seen in the Fig. 1, chromatin condensation and apoptotic nuclear fragmentation were observed by Hoechst staining (panels 1H and 2L) in influenza virus infected cells.

Table I Oligonucleotide sequences of the primers used in this study

Primer	Sequence 5′ to 3′	Sense
T7	TAA TAC GAC TCA CTA TAG G	+
T7-Uni12	TAA TAC GAC TCA CTA TAG GAG CAA AAG CAG G	+
vPB2qR3	TAG TAT CTC GCG AGT GCG AGA CT	–

Apoptosis of the Cells Infected

To confirm the presence of apoptosis and to make quantitative analysis, we examined the amount of ssDNA using ELISA at 18 h p.i.. At the 18 h p.i., the absorbance of ssDNA at 405 nm was 2.1 ± 0.05 for H8 cells and 1.95 ± 0.15 for Sw.71 cells. For mock treated cells and inactivated virus-treated cells, the absorbances of ssDNA were 0.28 ± 0.08 and 0.29 ± 0.11, respectively for H8 cells; and in the case of Sw.71 cells, they were 0.30 ± 0.07 and 0.38 ± 0.13, respectively. The absorbance values of negative and positive controls were 0.81 ± 0.01 and 2.68 ± 0.16, respectively. The amount of ssDNA which suggests presence of apoptosis induced by influenza infection of the two cell lines were significantly higher than those of the corresponding mock-treated cells and inactivated-virus treated cells ($P < 0.0001$ for all cases) (Fig. 2).

Viral Replication in Infected Cells into Culture Media

By HA assay, we observed the evidence of exocytotic release of the viral progeny from both cell lines. Although at 8 h p.i., HA titers were under detectable levels in both cell lines, they were detected at 24 h p.i. (Fig. 3). We observed almost identical results with plaque formation assay (data not shown). Real time RT-PCR detected viral RNA at 8 h p.i. in the both cell lines and significantly increased at 24 h p.i. (Fig. 4).

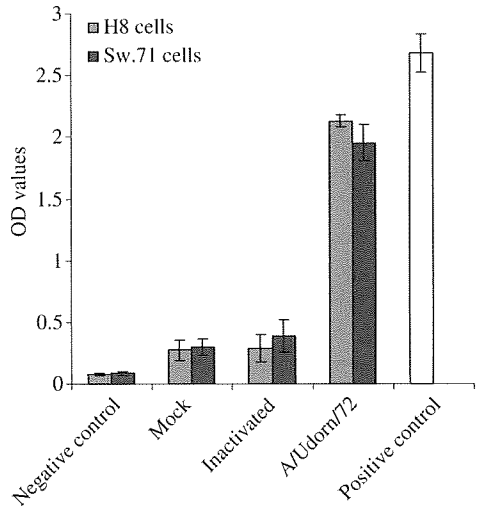

Fig. 2 ELISA detection of single-stranded DNA present in apoptotic cells. Each of the cell suspensions was prepared in a 96-well flat-bottom plate as specified by the manufacturer. Then the cells were inoculated with the influenza A/Udorn/72 H3N2 at an MOI of 5. Single-stranded DNA provided in the ELISA kit was used as a control. Results are expressed as the means and standard deviations of three determinations.

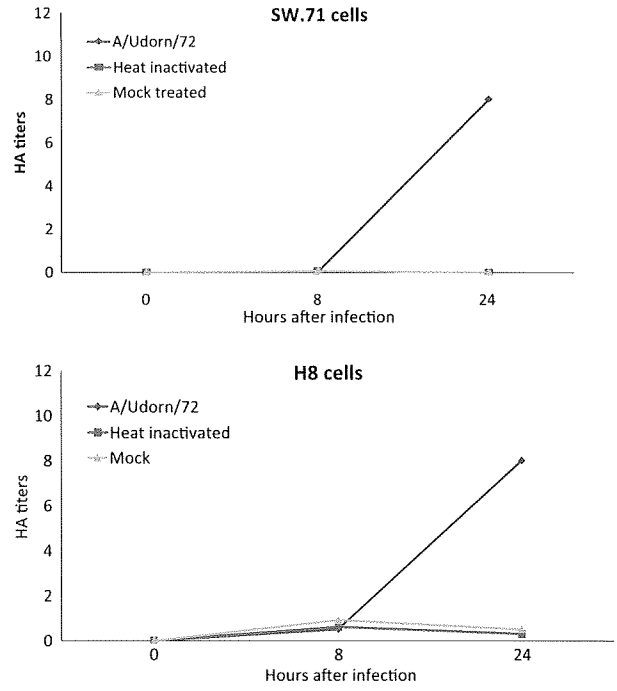

Fig. 3 Kinetics of influenza virus replication measured using the HA assay. H8 and Sw.71 cells were infected with the A/Udorn/72 at MOI 5. At the indicated time points post-infection (8 and 24 h p.i.), HA activity was measured in the culture supernatants.

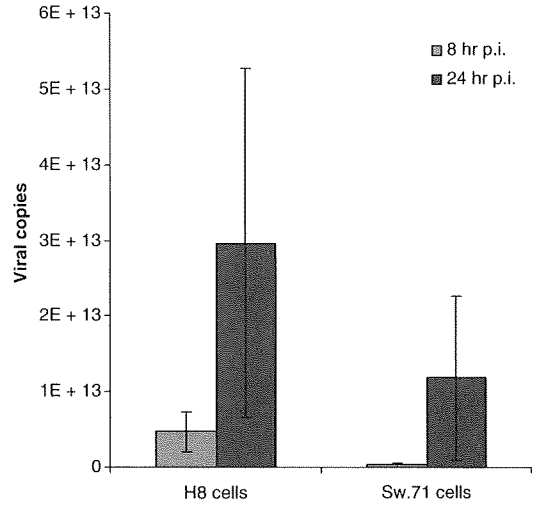

Fig. 4 Results of quantification of viral RNA copies in culture media using real-time PCR. *y*-axis: viral RNA copy numbers. The experiment was repeated three times. Each data point represents the mean ±S.D.

Expression of HLA-G

HLA-G is a non-classical major histocompatibility antigen with a restricted pattern of expression.[25] HLA-G is selectively expressed on cytotrophoblasts and invasive extravillous trophoblast at the feto-maternal interface where it may play a major role in maternal-fetal tolerance.[26] Its down-regulation caused by viral infections is considered to be a major cause of miscarriage.[27] We examined the expression of HLA-G using flow cytometry. Both in H8 and Sw.71 cells, we detected HLA-G in protein level. We observed no remarkable changes of HLA-G expression of the both cell lines at 8 and 24 h p.i. (Fig. 5). Forty-eight h p.i., we could detect no HLA-G protein because of cytopathic effects (data not shown).

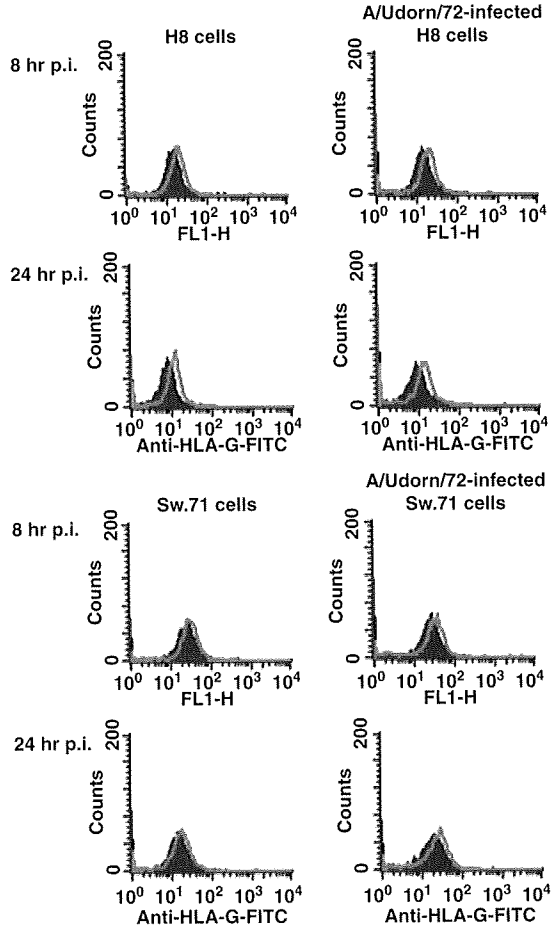

Fig. 5 Flow cytometry analysis of the non-classical class I MHC molecule HLA-G of H8 and Sw.71 cells by staining of HLA-G with FITC-conjugated mouse anti-human HLA-G monoclonal antibody. There was no shift in the histogram upon infection with the virus at 8 and 24 h p.i.

Discussion

In this study, we revealed that immortalized human trophoblast cell lines mimicking invasive trophoblasts are susceptible to H3N2 influenza virus. We consider that this finding is important because the human placenta is characterized by extensive invasion of cytotrophoblasts into the uterus wall, allowing their direct contact with the maternal blood, and by the extent of hormonal production.[28] In other words, trophoblastic invasion during the first trimester is a critical step to establish human pregnancy. Thus, insufficient invasion caused by various reasons including genetic abnormalities, disrupted maternal immune tolerance as well as viral infections results pregnancy failures. Productive infection and possible pathogenic roles of cytomegalovirus (CMV),[29] adenovirus,[30] adeno-associated virus-2 (AAV-2)[31] in early human trophoblasts have been reported while infection of influenza virus have not been studied despite its clinical importance. The most plausible explanation is that non-pandemic influenza is a relatively localized disorder in respiratory organs. Viremia is believed to occur infrequently in influenza.[32] A number of studies searching for influenza viremia after the onset of illness have failed to detect virus, supporting the notion that influenza viremia is at most a rare event in the post-symptomatic period and if it exists, it is not generally sustained for long periods. However, recent studies employing highly sensitive PCR suggested transient viremia before onset of respiratory is not rare.[33,34]

More importantly, viral RNA was detected from extrapulmonary sites including autopsy specimens of heart, kidney, brain, spinal cord, spleen, and liver of a pregnant 19-year-old woman who died as a result of A2/HongKong/8/68 infection.[35] Another case of a 24-year-old pregnant woman infected with influenza A/Bangkok (H3N2) showed positive results in fetal tissues and amniotic fluid.[36]

Taken together, these findings suggest fetoplacental tissues have a chance to be infected with influenza virus delivered by maternal systemic circulations. Limited viremia could be controlled with neutralizing antibodies evoked with the previous influenza infection or vaccinations.

However, in a case of pandemic, it is a completely different story. An influenza pandemic can develop with the emergence of a new virus with high transmission capability, and that harbors a novel HA that has not circulated for decades. In the 20th century, there were three overwhelming pandemics with

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influenza A, in 1918, 1957 and 1968, caused by H1N1 (Spanish flu), H2N2 (Asian flu) and H3N2 (Hong Kong flu), respectively. During pandemics, pregnant subjects might have a higher risk of viremia and subsequent transplacental infection because of lack of previous immune exposures.

Gu et al. reported an autopsy case of pregnant woman who was killed by H5N1 influenza.[37] Employing in situ hybridization and immunohistochemical methods, they observed positive staining of influenza virus in placenta and fetal organs. They speculated that in addition to the lungs, H5N1 influenza virus disseminates to other organs including the brain and could also be transmitted from mother to fetus across the placenta.

Taken together, our findings suggest not only pandemic H5N1 influenza virus but also H3N2 and possibly other seasonal influenza viruses might replicate effectively in the invasive trophoblasts and subsequently induce placental apoptosis which might cause congenital anomalies as well as pregnancy failures. This cytopathic effect is independent from reduced HLA-G expression often observed in placental viral infections.

Acknowledgments

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Association of CCR5-59029 A/G and CCL3L1 Copy Number Polymorphism with HIV Type 1 Transmission/Progression among HIV Type 1-Seropositive and Repeatedly Sexually Exposed HIV Type 1-Seronegative North Indians

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Abstract

The CCR5Δ32 mutation does not account for HIV-1 resistance in the majority of persons who are repeatedly exposed to HIV-1 by high-risk activities but remain seronegative and uninfected. Therefore, we investigated the impact of CCR5 59029 A/G and CCL3L1 copy number polymorphism on HIV-1 disease susceptibility and progression among HIV-1-infected and HIV-1-exposed seronegative North Indians. HIV-1-seropositive (HSP, $n = 196$) patients, stratified on the basis of disease severity (Stages I, II, and III) and HIV-1-exposed seronegative (HES, $n = 47$) individuals were genotyped for CCR5-59029 A/G polymorphism by PCR-RFLP and CCL3L1 copy number by the real-time TaqMan PCR method. A group of ethnically matched HIV-1-seronegative (HSN, $n = 315$) healthy volunteers were also genotyped as controls. Statistical analysis was done by SPSS software. The CCR5-59029 AG genotype was significantly higher in the HES compared with the HSP group (57.44% vs. 37.24%, $p = 0.014$). The CCL3L1 mean copy number of HES was higher compared with the HSP groups (3.148 ± 0.291 vs. 2.795 ± 0.122, $p = 0.212$), but was not significant when compared with independent samples t test. Possession of CCL3L1 copies ≤ 2 or >2 was not associated with enhanced or reduced risk of HIV-1 acquisition. Gene–gene interaction studies showed enrichment of the CCR5-59029AG*CCL3L1>2 genotype in the HES group when compared with the HSP group (31.91% vs. 15.81%, $p = 0.021$, OR = 0.401, CI = 0.194–0.826). The increased frequency of the CCR5-59029AG*CCL3L1>2 genotype among HES individuals led us to conclude that the CCR5-59029 AG genotype and CCL3L1 gene dose appeared to have synergistic or interactive effects and are expected to be involved in the host innate resistance to HIV-1 infection.

Introduction

Genetic susceptibility to HIV infection and AIDS progression is variable among individuals and populations. There are individuals who have been repeatedly exposed to HIV-1 but have remained HIV-1 uninfected. A subset of such HIV-1-exposed seronegative (HES) individuals is HIV-1-seronegative (HSN) spouses of HIV-1-seropositive (HSP) individuals who are sexually exposed to HIV-1 repeatedly, yet remain negative for anti-HIV antibody. Immunologic and genetic studies of these individuals have helped to elucidate protective mechanisms for HIV-1 infection.[1–4] The homozygosity for a mutation in the CCR5 gene (CCR5Δ32) is presently considered the most relevant against HIV-1 resistance,[5,6] which is rare among North Indians.[7–9] We have recently reported that in the absence of individuals having the CCR5Δ32 mutation, the DC-SIGNR 5/5 homozygous genotype may a have partial role in conferring HIV-1 resistance among these HES individuals.[10]

Among other host-dependent factors, attention has recently been paid to the variability in chemokine receptors and chemokines, which plays a central role in HIV-1 infection and disease pathogenesis. The principal chemokine receptor involved in HIV-1 transmission and progression is CCR5, and

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its most potent natural ligand is an MIP-1alpha (i.e., CCL3L1) chemokine.[11] An A/G polymorphism in the CCR5 promoter gene at nucleotide position 59029 was identified[12] and the CCR5-59029 GG homozygous genotype was reported to be associated with slower progression to AIDS.[12–14] The CCR5-59029 G polymorphism has also been linked to decreased surface expression and reduced *in vitro* infectability.[15] CCR5Δ32/wt in combination with the CCR5-59029 A/G genotype has been reported to have an advantage in resisting sexual HIV-1 transmission in white men.[16]

Independent studies have shown that an increased copy number of CCL3L1 results in the enhanced secretion of CCL3L1 by activated leukocytes.[17,18] CCL3L1 gene dosage has been reported to influence HIV-1 infection and pathogenesis. CCL3L1 copy numbers lower than the average for each ethnic group evaluated were associated with unfavorable outcomes, presumably due to lower expression and reduced capacity to block or interfere with HIV-1 binding to the CCR5 coreceptor.[17] Variations in the CCL3L1 copy number have been linked to HIV-1 susceptibility in asymptomatic Japanese patients with hemophilia,[19] while no such association was observed among HIV-1-infected North Americans.[20] The CCR5/CCL3L1 genotype combination also influences cell-mediated immunity (apart from viral burden to include viral entry-independent processes) in both healthy and HIV-1-infected individuals during the course of HIV infection.[21] Recent data also suggest that duplications of CCL3L1 are associated with the HIV-specific CD4 T cell responses that control HIV-1 infection.[22,23]

The relationship between CCR5/CCL3L1 and HIV-1 susceptibility and disease progression is in dispute and remains to be confirmed in other populations and settings. Given such evidence, it was reasonable to hypothesize that in the absence of the CCR5Δ32 mutation, the CCR5-59029A/G/CCL3L1 copy number polymorphism may confer a protective effect in HES individuals. Thus, in the absence of any study examining the relationships of CCR5 and CCL3L1 to HIV-1-related outcomes in HIV-1-infected and HIV-1-exposed seronegative North Indian individuals, the present study was undertaken.

Materials and Methods

Patient selection

A total of 196 HSP were enrolled from the outpatients attending the clinics of Sanjay Gandhi PostGraduate Institute of Medical Sciences, Lucknow, India from January 2004 to December 2006. The HSP 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 $CD4^+$ T cell counts and based on their 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. The 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), and Stage III ($n = 49$; symptomatic HIV-1 patients with AIDS in CDC category C1 to C3).[24,25] Only individuals who had a minimum follow-up time of at least 6 months were included in the study. A total of 315 age-matched normal healthy controls of similar ethnicity with HSN status were recruited for the present study. Forty-seven HSN individuals with a history of repeated sexual intercourse (twice a week) without use of any protection (e.g., condoms) with HIV-1-infected partners for at least 1 year were recruited in the HES group. HIV-1-seronegative status of the HES subjects was confirmed by Western blot at the regular interval of 3 months to 1 year. Demographic profiles of the study groups are given in Table 1. After informed consent, a 5-ml blood sample was taken in EDTA for analysis of DNA.

Determination of HIV-1 status and $CD4^+$ T cell counts

All individuals were screened for their HIV-1 status by primarily screening with ELISA (Vironostika, HIV Uni-FormII Ag/Ab, Biomerieux, The Netherlands) and subsequently confirmed with Western blot (LAV Blot I, Bio-Rad, France). $CD4^+$ T cell counts were 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 0.2–0.3 ml of peripheral whole blood using the QIAamp Blood kit (Qiagen, CA) according to the protocols supplied by the manufacturer. Usually ~0.1 μg genomic DNA was used for the genotyping studies.

Genotyping of CCR5 59029-G/A promoter polymorphism

Genotyping was done by restriction fragment length polymorphism PCR (RFLP-PCR) analysis as previously described.[12] Amplicons including the polymorphic site of interest were amplified from extracted genomic DNA using the

TABLE 1. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF NORTH INDIAN INDIVIDUALS CLASSIFIED ON THE BASIS OF HIV-1 SEROLOGICAL STATUS AND CENTERS FOR DISEASE CONTROL AND PREVENTION CRITERIA[a]

Subjects	*Age (median, range) in years*	*Sex (M/F ratio)*	*$CD4^+$ T cell counts/μl (median, range)*
HSN ($n = 315$)	58, 35–60	6/1	ND
HES ($n = 47$)	59, 35–65	7/1	ND
HSP ($n = 196$)	57, 30–67	8/1	145, 30–1304
Stage I ($n = 86$)[b]	58, 30–65	7/1	315, 65–1304
Stage II ($n = 61$)[b]	55, 35–60	9/1	175, 60–512
Stage III ($n = 49$)[b]	52, 38–67	8/1	120, 30–450

[a]HSN, HIV-1 seronegative; HES, HIV-1-exposed seronegative; HSP, HIV-1 seropositive; *n*, Number of individuals; M/F, male/female; ND, not detected.

[b]These groups were subgroups of the HIV-1-seropositive patient and were classified according to CDC (Centers for Disease Control and Prevention).[23,24] Clinical symptoms in Category A: asymptomatic HIV infection, persistent generalized lymphadenopathy (PGL); Category B: hairy leukoplakia, oropharyngeal candidiasis, vulvovaginal candidiasis, constitutional symptoms such as fever or diarrhea lasting >1 month, herpes zoster; Category C; *Pneumocystis carinii* pneumonia, *Mycobacterium tuberculosis* (pulmonary and disseminated), candidiasis of bronchi, trachea, or lungs, extrapulmonary cryptococcosis, cytomegalovirus disease (other than liver, spleen, or nodes), isosporiasis, chronic intestinal (>1 months duration), HIV-related encephalopathy, Kaposi's sarcoma, wasting syndrome due to HIV.

following primers: forward 5′-CCC GTG AGC CCA TAG TTA AAA CTC-3′ and reverse 5′-TCA CAG GGC TTT TCA ACA GTA AGG-3′. Of the PCR product 5 μl was digested with 4 units of Bsp1286I (MBI Fermentas) in a 20 (l volume at 37°C for 5 h using the buffer provided and bovine serum albumin. The digested product was loaded onto a 2% agarose gel, electrophoresed in a 1×TBE buffer, and stained with ethidium bromide.

Genotyping of CCl3L1 copy number by real-time PCR (TaqMan) assays

The CCL3L1 gene copy number was estimated by TaqMan assays using Rotorgene 3000 (Corbet Research, Australia) and primers/probes (Tibmolbio, Berlin, Germany) according to previously described method.[17] The CCL3L1 gene was amplified by using the following primer sequences: forward, 5′-TCT CCA CAG CTT CCT AAC CAA GA3′; reverse, 5′-CTG GAC CCA CTC CTC ACT GG-3′; and probe, 5′-FAM (6-carboxyfluorescein, 6-FAM)–AGG CCG GCA GGT CTG TGC TGA–TAMRA (6-carboxytetramethylrhodamine, TAMRA). The housekeeping gene, β-globin (HBB), served as the internal control and was amplified using primer sequences as follows: forward, 5′-GGC AAC CCT AAG GTG AAG GC-3; reverse, 5′-GGT GAG CCA GGC CAT CAC TA-3′; and probe 5′-VIC-CAT GGC AAG AAA GTG CTC GGT GCCT–TAMRA. Seven serial 1:2 dilutions (50–0.78 ng) of genomic DNA from human A431 cells, known to contain two copies of CCL3L1 per diploid genome (pdg) by Southern blot densitometry,[18] were used on each plate to generate standard curves of C_T (threshold cycle) value against the log [DNA] for HBB (also present at two copies) and the CCL3L1 gene. For each test sample, duplicate wells were set up for CCL3L1 and HBB. C_T value was determined, and converted into template quantity using the standard curves. Copy number is the ratio of the template quantity for CCL3L1 to the template quantity for HBB, multiplied by two. The amount of test DNA sample added to each PCR reaction was between 2 and 10 ng.

Sequencing

PCR-amplified products were randomly selected to be sequenced according to the manufacturer's 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). The direct gene counting method was used to determine the frequency of genotypes and alleles. The Fisher exact or χ^2 test was used to determine differences in allele/genotype frequencies of CCR5-59029 and copy number of CCL3L1 genetic variants. Association between the CCL3L1 mean copy number distributions among different groups was analyzed by independent samples *t* test. Odds ratios (OR) and its 95% confidence interval (CI) were obtained to describe the strength of association. A p value <0.05 was considered to be statistically significant. The sample size was calculated using the QUANTO ver. 1 program (http://hydra.use.edu/gxe). Calculated sample size was adequate to study both polymorphisms.

Results

CCR5-59029 A/G allele/genotype distribution analysis

Analysis of the genotype frequency data reveals significant deviation ($p < 0.05$) from HWE for the CCR5-59029 genotypes (Table 2). Deviation from expected values may be due to the excess of heterozygosity observed in CCR5-59029, which may be due to selection pressure. The CCR5-59029 AG genotype was significantly enhanced in the HES group compared to the HSP group (57.44% vs. 37.24%, $p = 0.014$) (Table 2). Further comparison of the CCR5-59029 A/G allele and genotype frequencies between Stage III and Stages I and II HIV-1-infected patients revealed no significant differences.

HIV-1/AIDS susceptibility and CCL3L1 copy number variations

Estimation of CCL3L1 copy number was done by the quantitative ratio of CCL3L1 and HBB in the template genomic DNA evaluated by the quantitative TaqMan PCR method (Fig. 1). The number of CCL3L1 copies ranged from 1 to 9 per diploid genome in the control cohort with the most common being 2. Thirty-two percent of the North Indian HIV-1-seronegative healthy controls carried two copies of the CCL3L1 gene. No statistically significant differences in distribution of the CCL3L1 copy number between the study groups were found (data not shown), consistent with previous studies[17,19] that reported that the individual copy numbers of the

TABLE 2. GENOTYPE FREQUENCIES OF CCR5-59029 A/G IN HIV-1-SERONEGATIVE, HIV-1-EXPOSED SERONEGATIVE, AND HIV-1-SEROPOSITIVE NORTH INDIANS[a]

Genotype	*HSP* (n = *196*)	*HSN* (n = *315*)	p *value*	*OR*	*95% CI*
AA	59 (30.10)	81 (25.71)	0.308	1.244	0.837–1.849
AG	73 (37.24)	123 (39.04)	0.709	0.926	0.641–1.338
GG	64 (32.65)	111 (35.23)	0.566	0.891	0.611–1.300
	HSP (n = *196*)	*HES* (n = *47*)	p *value*	*OR*	*95% CI*
AA	59 (30.10)	8 (17.02)	0.101	2.099	0.925–4.765
AG	73 (37.24)	27 (57.44)	0.014	0.44	0.230–0.839
GG	64 (32.65)	12 (25.53)	0.385	1.414	0.688–2.906
	Stage III (n = *49*)	*Stage I* (n = *86*)	p *value*	*OR*	*95% CI*
AA	18 (36.73)	26 (30.23)	0.451	1.340	0.639–2.811
AG	19 (38.77)	29 (33.72)	0.579	1.245	0.601–2.578
GG	12 (24.48)	31 (36.04)	0.184	0.575	0.262–1.263)
	Stage III (n = *49*)	*Stage II* (n = *61*)	p *value*	*OR*	*95% CI*
AA	18 (36.73)	15 (24.59)	0.210	1.781	0.782–4.054
AG	19 (38.77)	25 (40.98)	0.847	0.912	0.423–1.967
GG	12 (24.48)	21 (34.42)	0.299	0.618	0.267–1.429

[a]Data are no. (%) of subjects. AA, homozygous wild; AG, heterozygous; GG, homozygous mutant; CI, confidence interval; OR, odds ratio; HSN, HIV-1 seronegative; HES, HIV-1 exposed seronegative; HSP, HIV-1 seropositive. Stages I, II, and III are categorized on the basis of their CD4 counts and CDC classification. Statistical analysis: chi-square test by the SPSS statistical package.

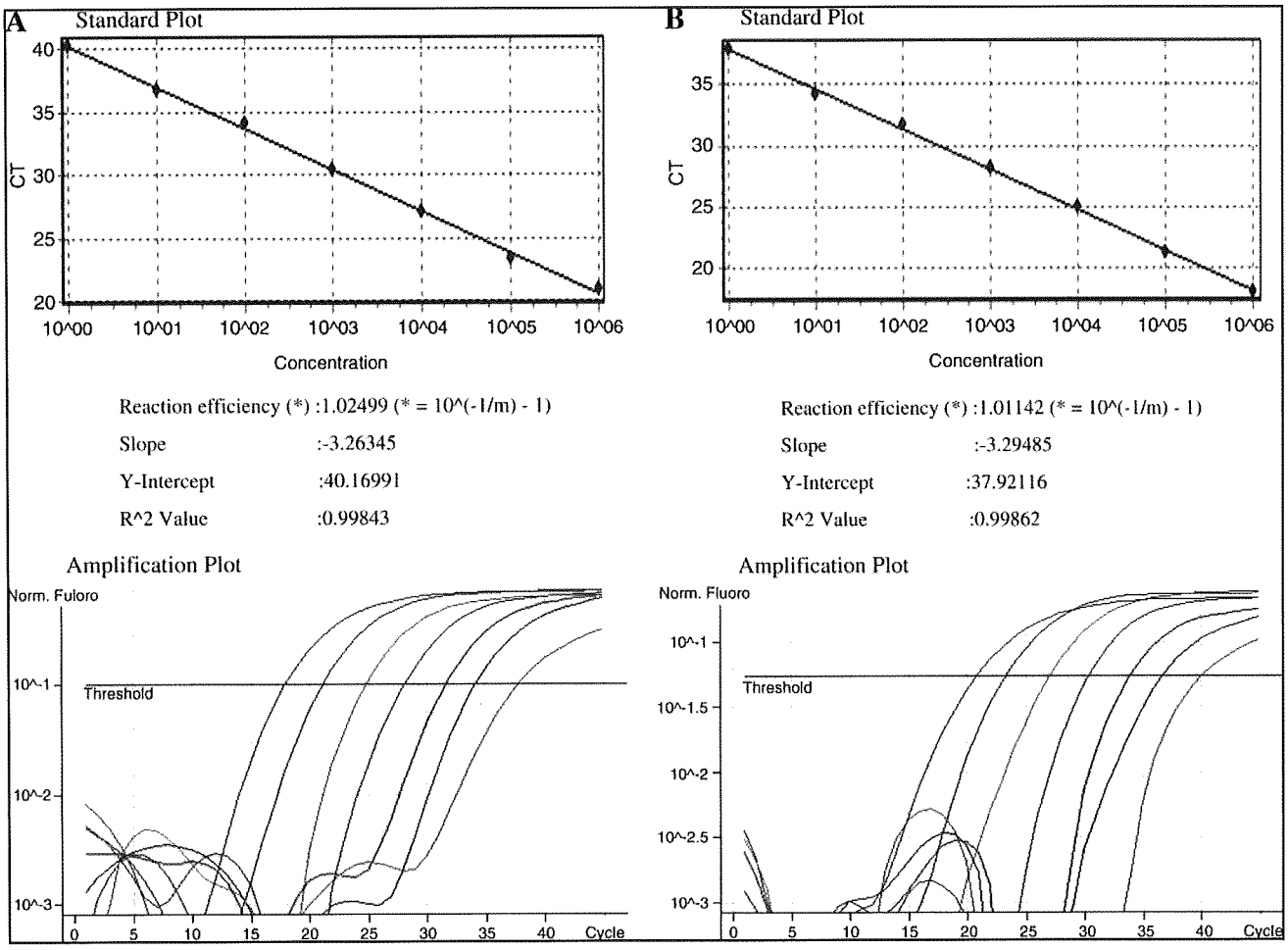

FIG. 1. Standard curve and amplification plots obtained by Rotor-Gene 6.0.33 software for the human β-globin gene (**A**) and CCL3L1 (**B**). The lower plots show the amplification curves obtained using seven (1:2) serial dilutions from 50 ng to 0.78 ng of human genomic DNA (cell line A431). The upper plot shows the standard curves with the slope and the square of the Pearson correlation coefficient (R^2) for the standard curves.

CCL3L1 gene are not associated with HIV/AIDS clinical outcomes. The CCL3L1 mean copy number of HES was, though higher, compared with the HSP groups (3.148 ± 0.291 vs. 2.795 ± 0.122, $p = 0.212$), but was not significant when compared with independent samples t test (Table 3). The median copy number in the HSN group was 2; therefore to test our hypothesis that a CCL3L1 copy number lower or higher than the median copy number influences the risk for HIV-1, subjects were divided into two groups: those with lower or equal than two (≤ 2) and higher than two (>2) copy numbers. Possession of CCL3L1 copies lower or equal to two (≤ 2) and higher than two (>2) was not associated with enhanced or reduced risk of HIV-1 acquisition (Table 4).

HIV-1/AIDS disease progression and CCL3L1 copy number variations

In the absence of number of years of known seroprevalence without antiretroviral therapy and viral load, the HSP group ($n = 196$) was categorized into Stages I, II, and III on the basis of their $CD4^+$ T cell counts and clinical CDC classification. There was no significant difference in the distribution of the CCL3L1 copy number (data not shown) and its mean among

TABLE 3. MEAN COPY NUMBER OF CCL3L1 GENES AMONG DIFFERENT STUDY GROUPS[a]

Groups	*Mean of CCL3L1 copy number*	*Median*	*SEM*	*SD*	p *value*
HSP (n = 196)	2.795	2	0.122	1.709	Reference
HSN (n = 315)	2.825	2	0.092	1.648	0.713[b]
HES (n = 47)	3.148	3	0.291	1.999	0.212[c]
Stage I ($n = 86$)	2.814	3	0.162	1.506	0.765[d]
Stage II ($n = 61$)	3.000	3	0.223	1.745	0.690[e]
Stage III ($n = 49$)	2.836	2	0.235	1.650	Reference

[a]HSN, HIV-1 seronegative; HES, HIV-1-exposed seronegative; HSP, HIV-1 seropositive; SD, standard deviation; SEM, standard error mean. Stages I, II, and III are categorized on the basis of their CD4 counts and CDC classification. Statistical analysis: independent-samples t test by the SPSS statistical package.
[b]HSP vs. HSN.
[c]HSP vs. HES.
[d]Stage III vs. I.
[e]Stage III vs. II.

Table 4. CCL3L1 Copy Number Polymorphism and HIV-1 Susceptibility/Progression[a]

CCL3L1 copy number	*HSP* (n = 196)	*HSN* (n = 315)	p *value*	*OR*	*95% CI*
CCL3L1≤2	103 (52.55)	164 (52.06)	0.928	1.020	0.714–1.457
CCL3L1>2	93 (47.44)	151 (47.93)	0.928	0.981	0.686–1.401
	HSP (n = 196)	*HES* (n = 47)	p *value*	*OR*	*95% CI*
CCL3L1≤2	103 (52.55)	22 (46.8)	0.518	1.259	0.665–2.382
CCL3L1>2	93 (47.44)	25 (53.19)	0.518	0.795	0.420–1.504
	Stage III (49)	*Stage I* (86)	p *value*	*OR*	*95% CI*
CCL3L1≤2	26 (53.06)	44 (51.16)	0.859	1.079	0.534–2.178
CCL3L1>2	23 (46.93)	42 (48.83)	0.859	0.927	0.459–1.871
	Stage III (49)	*Stage II* (61)	p *value*	*OR*	*95% CI*
CCL3L1≤2	26 (53.06)	33 (54.09)	1.000	0.959	0.451–2.038
CCL3L1>2	23 (46.93)	28 (45.90)	1.000	1.043	0.491–2.216

[a]Data are no. (%) of subjects. CI, confidence interval; OR, odds ratio; HSN, HIV-1 seronegative; HES, HIV-1-exposed seronegative; HSP, HIV-1 seropositive. Stages I, II, and III are categorized on the basis of their CD4 counts and CDC classification. Statistical analysis: chi-square test by the SPSS statistical package.

categorized HIV-1-infected patients (Table 3). CCL3L1 copies lower or equal than two (≤2) and higher than two (>2) were also not associated with HIV-1/AIDS disease progression when compared with HIV-1 seropositive patients classified into different stages on the basis of disease severity (Table 4).

Combined effects of CCR5-59029 genotypes and CCL3L1 median copy number

On the basis of genetic association data obtained for CCR5-59029 genotypes and population-specific CCL3L1 median copy number, i.e., 2, the study subjects were divided into six genetic risk groups: (1) CCR5-59029AA*CCL3L1≤2, (2) CCR5-59029AA*CCL3L1>2 (3) CCR5-59029AG*CCL3L1≤2, (4) CCR5-59029AG*CCL3L1>2, (5) CCR5-59029GG*CCL3 L1≤2, and (6) CCR5-59029GG*CCL3L1>2.

Thus, our working hypothesis was that a higher CCL3L1 copy number in association with a CCR5-59029 AG protective genotype might confer resistance in HES individuals against HIV-1 infection. Our data support the presence of an interaction between CCR5-59029 and CCL3L1 in the HES cohort only (Table 5). The frequency of CCR5-59029AG*CCL3L1>2 was significantly increased in the HES group when compared with the HSP group (31.91% vs. 15.81%, $p = 0.021$). Further comparison of CCR5-CCL3L1 genotypes between HIV-1-infected patients classified on the basis of severity of disease (Stages I, II, and III) did not show any significant association (Table 5). Further stratification of the groups by age and gender did not provide evidence of an association between CCR5 promoter alleles/genotypes and CCL3L1 copy number with HIV infection and progression (data not shown).

Discussion

Resistance to HIV-1 infection in some individuals has generated biological and genetic differences in explaining differential susceptibility to HIV-1 infection. Genetic variability in chemokine receptor genes and their ligands plays a major role in providing susceptibility to HIV-1 transmission and disease progression.

In the present study, we have provided evidence that the CCR5-59029 A/G polymorphism influences HIV-1 resistance among HIV-1-exposed seronegative North Indian individuals. The CCR5-59029 AG genotype was significantly enriched in HIV-1-exposed seronegative individuals, consistent with the previous studies in white men.[16] In contrast to previous studies,[12–14,25] the inability of the present study to find any association between CCR5-59029 A/G polymorphism and delayed disease progression is most likely due to differences in ethnicity and environmental and social conditions of the populations. Our results were consistent with previous studies in a London cohort that failed to find any disease-retarding effect of the G nucleotide.[26] Our results were also consistent with the previous studies on North Indians[7] and white Europeans[27] that failed to show any significant association of the CCR5-59029 A/G polymorphism with disease progression among HIV-1-positive individuals. The present study further elucidated a possible genetic variant other than DC-SIGNR, previously reported in the same cohort,[10] that is operative in conferring resistance to HIV-1 infection among HIV-1-exposed seronegative North Indian individuals.

Results from our study demonstrated that 32% of the North Indian healthy controls have two copies of the CCL3L1 gene. It was lower than New Zealand controls (51%),[28] United Kingdom controls (53%),[28] European Americans (49%),[17] and white U.S. subjects (51%)[18] and higher than African Americans (16%)[20] and Japanese (10%).[19] Gonzalez *et al.*[17] reported

Table 5. Comparison of CCR5-59029/CCL3L1 Combined Genotypes between HIV-1-Seropositive and HIV-1-Exposed Seronegative North Indians[a]

Combined genotypes	*HSP* (n = 196)	*HES* (n = 47)	p *value*	*OR*	*95% CI*
CCR5-59029AA*CCL3L1<2	22 (11.22)	3 (6.38)	0.429	1.854	0.531–6.478
CCR5-59029AA*CCL3L1>2	37 (18.87)	5 (10.63)	0.205	1.955	0.724–5.281
CCR5-59029AG*CCL3L1<2	42 (21.42	12 (25.53)	0.56	0.795	0.380–1.666
CCR5-59029AG*CCL3L1>2	31 (15.81)	15 (31.91)	0.021	0.401	0.194–0.826
CCR5-59029GG*CCL3L1<2	39 (19.89)	7 (14.89)	0.536	1.419	0.591–3.409
CCR5-59029GG*CCL3L1>2	25 (12.75)	5 (10.63)	0.809	1.228	0.444–3.398

[a]Data are no. (%) of subjects. AA, homozygous wild; AG, heterozygous; GG, homozygous mutant; CI, confidence interval; OR, odds ratio; HES, HIV-1-exposed seronegative; HSP, HIV-1 seropositive. Statistical analysis: chi-square test by the SPSS statistical package.

that individuals with <2 copies of the CCL3L1 gene from the median for their population group presented a significantly higher risk for acquiring HIV-1 infection, whereas those with >2 copies above the mean had significantly less risk. Moreover, a gene dose lower than the cohort median was associated with increased risk of progressing more rapidly to AIDS and death. Similar findings by Nakajima *et al.*[19] also reported that the average copy number of CCL3L1 in hemophilic HIV-1-infected subjects was significantly lower than in controls and subjects possessing two or fewer copies of CCL3L1 had a significantly higher risk of acquiring HIV-1.

Our results, however, do not support this hypothesis, showing equal frequency of CCL3L1 copy number variants in control and HIV-1-infected groups. This is in agreement with other groups, which failed to find any significant association of CCL3L1 copy number with HIV-1 susceptibility/progression.[20] Moreover, Nakajima *et al.* also reported that CCL3L1 copy number variations had no significant effect on the disease progression among the long-term nonprogressor (LTNP) subjects, when compared with nonprogressors and patients under treatment.[19] Analysis of HIV-infected European individuals also failed to detect any statistically significant association between the distribution of CCL3L1 gene copy number and rate of progression to AIDS.[18] In the absence of finding any association between CCL3L1 copy number and HIV/AIDS susceptibility it can be speculated that expression of CCL3L1 mRNA may be independent of CCL3L1 gene copy number and may be regulated/influenced by other factors.[29] The discrepancy with respect to CCL3L1 gene dose and HIV-1 susceptibility between the present study and previous studies of Gonzalez *et al.*[17] and Nakajima *et al.*,[19] though not clear, may be due to differences in racial or ethnic background, study design, and prevalence of virus subtype. The absence of number of years of known HIV-1 seropositivity could also be another factor that may influence disease progression in these patients. The integrity of the immune system, the antiretroviral schedules, the compliance with the therapeutic regimen, and the presence of the reactivation of chronic infections in HIV-1-infected patients classified as Stage III could also influence disease progression and could also explain the contradictory results obtained in different studies.

Investigating the association between possession of CCR5-59029/CCL3L1 combined genotypes and risk of acquiring HIV and/or disease progression, a higher frequency of CCR5-59029AG*CCL3L1>2 was observed in HIV-1-exposed seronegative compared to HIV-1-seropositive patients. Thus, the CCR5-59029 genotype and CCL3L1 gene dose appeared to have synergistic or interactive effects involved in the host innate resistance to HIV-1 infection. The CCL3L1 gene copy number is not the absolute parameter that confers HIV/AIDS susceptibility[29]; rather it acts synergistically with other host factors. It is conceivable that the CCR5-59029 A/G and CCL3L1 copy number polymorphism evaluated in this study may potentially exert additive or synergistic influence with other factors, such as the antiviral immune response mediated by the $CD8^+$ T cells and the levels of β-chemokines, the differential coreceptor expression on target cells, human leukocyte antigens (HLA), and cytokine polymorphisms.[30,31] Moreover, cellular factors such as cytosine deaminases APOBEC3G (*apo*lipoprotein *B* mRNA-*e*diting enzyme, *c*atalytic polypeptide-like 3G), protease inhibitors, α_1-antitrypsin, and viral factors such as subtypes and the viral load presented either by the sexual partners of the exposed but uninfected individuals or by the infected individuals enrolled in this study could also exert some effects on the risk of acquisition and disease progression, respectively.[32] In addition, there are likely to be as yet unidentified host factors that are important in determining the outcome of HIV-1 infection. The protective effect of the CCR5-59029AG*CCL3L1>2 genotype against HIV-1 infection is further supported by evidence that reported an inverse association between CCL3L1 copy number and CCR5 expression on the T cell surface.[17] Recent studies have shown the presence of β-chemokine-mediated resistance against HIV-1 infection among HIV-1-exposed but -uninfected partners of HIV-1-infected individual.[30]

Numerous studies in the past have provided important insight regarding the single nucleotide polymorphisms (SNPs) in host genetic factors influencing the susceptibility and resistance to HIV-1 infection. However, it is very difficult to attribute the effect of a particular gene, which could explain, by itself, the natural resistance to HIV-1 infection among sexually exposed but seronegative individuals and the disease progression in HIV-1-infected individuals. This is due to the fact that much of the genetic variation underlying infectious disease susceptibility is complex, involving the combination of many loci, as is further confounded by SNP variations between different populations and ethnic groups. However, further studies are required to confirm the present findings, correlating the MIP1-alpha concentration in serum samples with CCL3L1 gene copy number, and CCR5 expression on PBMCs with HIV-1 susceptibility in larger and well-established cohorts.

In conclusion, in the absence of an HIV-1 resistance conferring mutation, i.e., CCR5 delta 32, resistance to sexual transmission of HIV-1 infection among HIV-1-exposed seronegative individuals might be conferred by a synergistic effect of the CCR5-59029 AG genotype and CCL3L1 gene. The overall understanding of the molecular mechanisms underlying resistance to viral infection remains elusive and their elucidation may offer valuable clues for the development of HIV therapeutics and vaccines, providing a basis for the prevention of HIV/AIDS.

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Disclosure Statement

No competing financial interests exist.

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Short communication

Evolutionary consequences of G9 rotaviruses circulating in Thailand

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ABSTRACT

Infection with group A rotaviruses is the main cause of acute gastroenteritis in infants and young children worldwide. Rotavirus G9 is recognized as the fifth most predominant G genotype that spreads throughout the world. In this study, we describe the changing distribution of rotavirus G9 genotype in Chiang Mai, Thailand, from 1989 to 2005. Molecular genetic evolutionary analysis of the G9 rotavirus VP7 gene was performed. The G9 rotavirus in Thailand was first detected in Chiang Mai in 1989 with a low prevalence of 1.98%, but in 2000 and 2001 it had become the most predominant genotype, reaching prevalence of 75% and 92.2%, respectively. Then, the prevalence of G9 reached a peak of 100% in 2002 and decreased abruptly over the next 3 years, i.e. 16.7% in 2003, 32.1% in 2004, and 4.7% in 2005. Our phylogenetic analysis demonstrates that all the G9 rotaviruses circulating in Thailand belonged to lineage III, and clustered closely together based on the year of virus isolation. One amino acid change from Thr to Ile was observed in antigenic region C at position 208 between the G9 strains isolated in 1989 and the strains of 1997–2005. These findings provide the overall picture and genomic data of G9 rotaviruses circulating in Chiang Mai, Thailand.

1. The study

Group A rotaviruses are the most important etiologic agent of gastroenteritis and severe diarrhea in infants and young children (Estes and Kapikian, 2007). Rotaviruses are member of the *Reoviridae* family. The viruses contain two outer capsid proteins, VP4 and VP7, which allow classification of the rotaviruses into P (Protease-sensitive) and G (Glycoprotein) genotypes, respectively. Based on both antigenic and genetic differences, at least 23 distinct G genotypes and 31 P genotypes have been identified (Khamrin et al., 2007a; Steyer et al., 2007; Matthijnssens et al., 2008; Schumann et al., 2009; Ursu et al., 2009). Epidemiological studies around the world have demonstrated that rotavirus strains bearing the G and P combinations of G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], and G9P[6] are responsible for most rotavirus infections (Santos and Hoshino, 2005). The G9 rotavirus was first reported in the United States in the early 1980s (Clark et al., 1987). Thereafter, 9 disappeared for about a decade and then reemerged by the end of the 1980s, and currently it represents the fifth most common G genotype of rotavirus infections throughout the world (Gentsch et al., 2005; Santos and Hoshino, 2005; Khamrin et al., 2006). Recent retrospective study of the stool specimens collected in 1980 from the United States revealed three specimens positive for rotavirus G9P[8] genotype, which represented the earliest human G9 viruses ever isolated (Cao et al., 2008).

In Thailand, two G9 rotavirus strains (Mc323 and Mc345) were first detected in 1989 in Chiang Mai province at a relatively low prevalence in children hospitalized with diarrhea (Urasawa et al., 1992), and both of them were in combination with P[19] (Okada et al., 2000). Later, it was reported again in 1996–1997 and 2000–2004 (Zhou et al., 2001; Khamrin et al., 2006, 2007b). It is interesting to note that G9 strains isolated in 1996–1997 were associated with either P[4] or P[6] genotypes, while the 2000–2004 strains were exclusively associated with the P[8] genotype. Molecular epidemiological studies of rotaviruses show the diversity of the specific genotypes found in humans in different parts of the world (Santos and Hoshino, 2005). Therefore, continuous monitoring of rotavirus genotype in different regions around the world is essential for the development of efficacious rotavirus vaccine. Genetic and antigenic drift is one of the major driving forces of rotavirus evolution (Iturriza-Gómara et al., 2004; Phan et al., 2007; Trinh et al., 2007). Therefore, the objectives of the present study were to detect and characterize G9 rotavirus strains circulating in Chiang Mai, Thailand in the year 2005 and determine their genetic relationship with G9 strains reported earlier from the same and other regions of the world.

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