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Anti-Retroviral Drug Resistance-Associated Mutations Among Non-subtype B HIV-1-Infected Kenyan Children With Treatment Failure

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Recently increased availability of anti-retroviral therapy (ART) has mitigated HIV-1/AIDS prognoses especially in resource poor settings. The emergence of ART resistance-associated mutations from non-suppressive ART has been implicated as a major cause of ART failure. Reverse transcriptase inhibitor (RTI)-resistance mutations among 12 non-subtype B HIV-1-infected children with treatment failure were evaluated by genotypically analyzing HIV-1 strains isolated from plasma obtained between 2001 and 2004. A region of *pol-RT* gene was amplified and at least five clones per sample were analyzed. Phylogenetic analysis revealed HIV-1 subtype A1 (n = 7), subtype C (n = 1), subtype D (n = 3), and CRF02_AG (n = 1). Before treatment, 4 of 12 (33.3%) children had primary RTI-resistance mutations, K103N (n = 3, ages 5–7 years) and Y181C (n = 1, age 1 year). In one child, K103N was found as a minor population (1/5 clones) before treatment and became major (7/7 clones) 8 months after RTI treatment. In 7 of 12 children, M184V appeared with one thymidine-analogue-associated mutation (TAM) as the first mutation, while the remaining 5 children had only TAMs appearing either individually (n = 2), or as TAMs 1 (M41L, L210W, and T215Y) and 2 (D67N, K70R, and K219Q/E/R) appearing together (n = 3). These results suggest that "vertically transmitted" primary RTI-resistance mutations, K103N and Y181C, can persist over the years even in the absence of drug pressure and impact RTI treatment negatively, and that appearing patterns of RTI-resistance mutations among non-subtype B HIV-1-infected children could possibly be different from those reported in subtype B-infected children. ***J. Med. Virol. 79:865–872, 2007.*** © 2007 Wiley-Liss, Inc.

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INTRODUCTION

The emergence of anti-retroviral drug (ARV)-resistance mutations is a major cause of anti-retroviral treatment (ART) failure [D'Aquila et al., 1995; Lorenzi et al., 1999; Zolopa et al., 1999]. These drug-resistant HIV-1 strains can be transmitted through vertical, sexual, and parenteral routes [Erice et al., 1993; Conlon et al., 1994; Boden et al., 1999; Little et al., 1999; Brenner et al., 2000; Pillay et al., 2000; Salomon et al., 2000; Duwe et al., 2001]. Vertically transmitted multi-drug resistant HIV-1 strain has been shown to persist for 9 months in an infant after postnatal therapy [Johnson et al., 2001]. Similarly, K103N-containing HIV-1 variants acquired after the administration of single dose-nevirapine, a non-nucleoside reverse-transcriptase inhibitor (NNRTI), have been reported to persist for more than 1 year in some women and infants after vertical transmission [Flys et al., 2005]. However, long-term persistence of vertically

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transmitted ARV-resistance mutations in the absence of drug pressure among infants and children is yet to be demonstrated.

Recently, the importance of ARV-resistant strains detected as minor populations has been reported. Minor drug-resistant HIV-1 populations have been detected both in the early phase of treatment failure [Coffin, 1995] and during successful structured treatment interruption [Metzner et al., 2003]. Minor drug-resistant populations undetectable by conventional assays can eventually overgrow and affect the clinical course [Dykes et al., 2004; Lecossier et al., 2005]. These minor drug-resistant populations have also been found to persist longer than expected previously in untreated patients, a favorable condition for wild-type virus to overgrow, which also indicates the risk of resistance transmission even from minor strains [Charpentier et al., 2004].

In patients experiencing treatment failure with nucleoside reverse-transcriptase inhibitors (NRTI), such as lamivudine plus either zidovudine or stavudine, the M184V mutation has been reported to always appear first, eventually followed by cumulative acquisition of thymidine-analogue-associated mutations (TAMs) if treatment with non-suppressive regimen is continued [Johnson et al., 2005]. Extensive studies on ARV-resistance suggest that HIV-1 may develop TAMs by either one of two distinct pathways; TAM 1 (M41L, L210W, and T215Y) or TAM 2 (D67N, K70R, and K219Q/E/N/R) [Flandre et al., 2003; Cozzi-Lepri et al., 2005]. However, most of these studies have focused on HIV-1 subtype B, which accounts for only 12% of the global HIV/AIDS pandemic, and data on non-subtype B HIV-1 is still limited. Furthermore, several differences in the development of ARV-resistance between subtype B and non-subtype B HIV-1 have been suggested [Apetrei et al., 1998; Quinones-Mateu et al., 1998; Pieniazek et al., 2000]. Most ARV-resistance studies have focused on adult populations [Yerly et al., 1998; de Ronde et al., 2001; Dykes et al., 2001; Brenner et al., 2002; Wainberg, 2003]. However, these findings may not be applicable directly to children, since several factors influencing selection of ARV-resistance such as pharmacokinetic properties; drug safety, tolerance, and antiviral activity of combination therapy, are usually different in the children [Kline et al., 1996].

The aim of this study was to investigate the patterns of emergence and the variable stability of ARV-resistance-associated mutations among non-subtype B HIV-1-vertically-infected children who developed eventually clinical failure with subsequent ART.

METHODS

Study Population

The subjects in this study resided in children's home in Nairobi, which housed 95 HIV-1-infected children. These children were born to HIV-1-infected mothers who either died of, or were too debilitated by HIV/AIDS hence could not offer basic care to the children. Of 95 children 55 were on ART as of August 2004. The duration of ART varied among children (mean: 23.3 months, range: 5–46 months). Of 55 children on ART 12 (8 males and 4 females, mean age: 7.4 years) experienced treatment failure, characterized by an initial decrease in plasma viral load (to undetectable level in one child) after treatment initiation and subsequent increase in the viral load as treatment continued. Seven of the 12 children received single ART regimen only during the study period: 5 received zidovudine/lamivudine/nevirapine, 1 zidovudine/didanosine/efavirenz, and 1 zidovudine/lamivudine/efavirenz (Table I). On the other hand, the remaining five children received multiple ART regimen during the study period: two received zidovudine/lamivudine/efavirenz followed by zidovudine/didanosine/efavirenz, two zidovudine/lamivudine/nevirapine followed by didanosine/lamivudine/efavirenz, and one didanosine/lamivudine/abacavir followed by zidovudine/didanosine/efavirenz and later didanosine/stavudine/efavirenz (Table I). These 12 children were admitted into the home by their first birthday and their HIV-1 status was confirmed serologically at 18 months of age. None of these children had history of previous exposure to any ARV.

This study was approved by the Kenya Medical Research Institute's National Ethical Review Committee on behalf of the Kenyan Government and conducted according to the national and international regulations governing the use of human subjects in biomedical research. The study was conducted within the continuing anti-retroviral, medical and healthcare programs of the institution without additional demand for blood samples solely for research purposes.

$CD4^+$ Cell Counts and Plasma Viral Loads

$CD4^+$ T cell counts of peripheral blood were determined using the FACSCOUNT (Becton-Dickinson, Beiersdorf, Germany) and plasma HIV-1 RNA loads using the Amplicor HIV-1 Monitor kit version 1.5 (Roche Diagnostics, Alameda, CA) with detection limit of 400 copies/ml according to the manufacturer's instructions.

Extraction and Amplification of Plasma HIV-1 Viral RNA

HIV-1 RNA was extracted from 100 μl of plasma using SMITEST EX-R and D (Sumitomo Metal Industries, Tokyo, Japan) according to the manufacturer's instructions. A region of the *pol-RT* gene (corresponding to nt 2480–3180 of HIV-1_{HXB2}) was amplified by both one-step RT-PCR (Invitrogen, Carlsbad, CA) and nested PCR with primer pairs, RT18 (5′-GGAAACCAAAAATGATAGGGGGAATTGGAGG-3′) and KS104 (5′-TGACTTGCCCAATTTAGTTTTCCCACTAA-3′) in the first round, and KS101 (5-GTAGGACCTACACCTGTTCAACATAATTGGAAG-3) and KS102 (5′-CCCATCCAAAGAAATGGAGGAGGTTCTTTCTGATG-3′) in the second round [Ndembi et al., 2004; Songok et al.,

TABLE I. General Characteristics of Non-B Subtype HIV-1-Infected Study Children

Sample ID	Age* (years)/sex	HIV-1 subtype/CRF	Study point (month, year)	ART[a] (initiation time)	$CD4^+$ T cell count (/μl)	Plasma viral load (copies/ml)	NRTI[b]-resistance mutations	NNRTI[c]-resistance mutations
NYU30	11/F	A1	Jul '02	ZDV, 3TC, EFV (Jun '01)	456	<400		
			Mar '03		475	24,857		
			Jan '04	ZDV,DDI,EFV (May 03)	267	89,063	*D67N + K70R + K219Q*	*L100I*
NYU33	11/F	A1	Jul '02	ZDV, 3TC, EFV (Jun '01)	549	3,449		
			Mar '03	ZDV,DDI,EFV (Oct 01)	556	122,419	*K219Q*	*K101Q*
			Feb '04		690	6,457	*K219Q + D218E*	*K101Q*
NYU36	11/M	D	Oct '01	ddI,3TC,ABC (Apr '01)	309	114,754	*M184V + T215F*	
			May '02			880,405	*M184V + T215F*	*I178M*
			Aug '02	ZDV,DDI,EFV (Oct 01)	321	81,870	*M184V + T215F*	*G190A*
			Apr '03		279	607,224	*T215F*	*G190A*
			Feb '04	D4T,DDI,EFV (Nov 02)	458	393,420	*T215F*	*G190A*
NYU38	10/M	C	Mar '03	ZDV,3TC,NVP (Sep '02)	388	38,459	*D67N*	
			Dec '03		188	60,695	*D67N + K70R + L210W + K219E*	
			Feb '04	DDI,3TC,EFV (Mar 04)	157	38,211	*D67N + K70R + L210W + K219E*	
			Aug '04		149		*D67N + K70R + L210W + D218E + K219E*	
NYU44	9/M	A1	Feb '02		208	1,017,931		*K103N*
			Mar '03	ZDV, DDI, EFV (May '02)	370	71,895	*D67N + K70R + T215F + K219Q*	*K103N + G190A*
			Dec '03		474	150,549	*D67N + K70R + T215F + K219Q + M41L + V75M*	*K103N + G190A*
NYU62	8/M	A1	Dec '01		589	239,644		
			Sep '02	ZDV, 3TC, NVP (Sep '02)	828	2,838	*D67N + K70R*	*G190A*
			Mar '03		568		*D67N + K70R + T215F + K219E*	G190A
			May '04			6,901	*D67N + K70R + T215F + K219E*	*G190A + Y181C*
NYU69	6/M	A1	Mar '03		192	227,176		*K103N*
			May '04	ZDV, 3TC, NVP (Mar '03)	400	113,868	*M184V*	*K103N*
NYU70	7/M	D	Sep '02		718	700,563		*K103N*
			Jun '03	ZDV, 3TC, NVP (Jul '03)	169	1,323,431		*K103N*
			Dec '03		502	188,059	*K70R + M184V*	*K103N*
NYU79	6/M	A1	Feb '03	ZDV,3TC,NVP (Apr '03)	70	159,826		
			Feb '04		551	244,506	*V75M + M184V*	*K101E + G190A*
			Jun '04	DdI,3TC,EFV (Mar 04)	347	472,203	*V75M + M184V*	*K101E + G190A + Y181C*
NYU83	5/M	A1	May '01		876	634,644		
			Jul '02	ZDV, 3TC, EFV (May '04)	946	50,570	*M184V*	*K103N*
			Apr '03		1138	74,437	*M184V*	*K103N*
			Aug '04		1125	197,301	*M184V + T215Y*	*K103N*
NYU85	5/F	CRF02_AG	Feb '03		178	30,690		
			Dec '03	ZDV, 3TC, NVP (Apr '03)	1214	3,264	*D67N + M184V*	*K103N*
			Apr '04		1148	79,080	*D67N + M184V*	*K103N*
NYU90	2/F	D	Apr '03		6	523,950		*Y181C*
			Jan '04	ZDV, 3TC, NVP (Apr '03)	399	55,679	*M184V*	
			Mar '04		379	155,191		*K103N*

*As of August 2004.
[a]ART, anti-retroviral therapy; ZDV, zidovudine; ddI, didanosine; EFV, efivarenz; NVP, nevirapine; 3TC, lamivudine; d4T, Stavudine.
[b]NRTI, nucleoside analogue RTI.
[c]NNRTI, non-nucleoside RTI; blank, no mutation detected.

2004]. Amplification was done with 1 cycle of 95°C for 10 min and 35 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, with a final extension of 72°C for 10 min. PCR amplification was confirmed by ethidium bromide staining of samples electrophoresed on an agarose gel.

Cloning, Sequencing, and Subtyping

The amplified products were cloned using the TOPO TA Cloning kit (Invitrogen) and sequenced as described previously [Ndembi et al., 2004; Songok et al., 2004]. The sample nucleotide sequences were aligned with HIV-1 subtype reference sequences from the Los Alamos database by CLUSTALW (version 1.81) with minor manual adjustments. Phylogenetic trees were constructed and visualized as described previously [Ndembi et al., 2004; Songok et al., 2004]. To improve the accuracy of HIV-1 subtyping, we used the genotyping tool (http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi), and the REGA subtyping tool (http://dbpartners.stanford.edu/RegaSubtyping/) as needed.

RTI Resistance-Associated Mutations

The RT nucleotide sequences (697 bps) were translated into the corresponding 232 amino acids and analyzed for previously reported drug resistance-associated mutations in subtype B strains using the Stanford university HIVdb sequence analysis program. For each sample, at least five clones were obtained and genotyped to detect the presence of minor populations.

RESULTS

General characteristics, treatment history, demographic, immunological, and virological data of the 12 HIV-1-infected children studied are summarized in Table I.

HIV-1 Subtypes

All children were infected with non-subtype B HIV-1: subtype A1 (n = 7), subtype C (n = 1), subtype D (n = 3), and circulating recombinant form (CRF)-02_AG (n = 1) (Table I).

RTI Resistance-Associated Mutations Before Treatment

Of the 12 children, 4 (33.3%) harbored NNRTI-resistance mutations before treatment. Three children, NYU44 (age, 7 years), NYU69 (5 years), and NYU70 (6 years), had K103N while NYU90 (1 year) had Y181C detected before treatment (Table I). All the mutations but one (one of seven clones in NYU69) were detected as full clones (Table IV). K103N detected in three children persisted, while Y181C detected in one child disappeared during treatment.

Emerging Pattern of NRTI Resistance-Associated Mutations

The patterns of NRTI-resistance mutations are summarized in Table II. M184V appeared as the first

TABLE II. Patterns of NRTI[a]-Resistance Mutations in Non-B Subtype HIV-1-Infected Children With Treatment

Child (ID)	Study point (mpti[a])					Treatment
	1st	2nd	3rd	4th	5th	
NYU69	*M184V* (10)					ZDV/3TC
NYU90	*M184V* (9)					ZDV/3TC
NYU83	*M184V* (13)	*M184V* (22)	*M184V* + 1TAM[b] (38)			ZDV/3TC
NYU70	*M184V* + 1TAM (6)					ZDV/3TC
NYU85	*M184V* + 1 TAM (9)	*M184V* + 1TAM (12)				ZDV/3TC
NYU36	*M184V* + 1 TAM (6)	*M184V* + 1 TAM (13)	*M184V* + 1TAM (18)	1 TAM (24)	1 TAM (34)	DDI/3TC/ABC, ZDV/DDI, D4T/DDI
NYU62	2 TAMs (6)	4 TAMs (12)	4 TAMs (22)			ZDV/3TC
NYU44	4 TAMs (11)	5 TAMs + *V75M* (19)				ZDV/DDI
NYU33		1 TAM (23)	2 TAMs (34)			ZDV/3TC, ZDV/DDI
NYU30			3 TAMs (31)			ZDV/DDI
NYU38	1 TAM (8)	4 TAMs (15)	4 TAMs (17)	5 TAMs (23)		ZDV/3TC, DDI/3TC
NYU79	*M184V* + *V75M* (10)	*M184V* + *V75M* (13)				ZDV/3TC, DDI/3TC

[a]NRTI, nucleoside analogue RTI.
[a]mpti, months post treatment initiation.
[b]TAM, thymidine analogue-associated resistance mutation; blank, no mutation detected.

primary NRTI-resistance mutation in 3 of 12 children (NYU69, NYU90, and NYU83), (later followed by the acquisition of one TAM in NYU83), while M184V appeared as first primary NRTI-resistance mutation with one TAM in three children (NYU36, NYU70, and NYU85) who received zidovudine/lamivudine, zidovudine/didanosine, or lamivudine/didanosine. The remaining five children (NYU30, NYU33, NYU38, NYU44, and NYU62) had a mixture of TAMs appearing as first mutations. Three of them (NYU44, NYU62, and NYU38) had both TAM 1 (M41L, L210W, and T215Y) and TAM 2 (D67N, K70R, and K219Q) profiles detected together. M184V appeared as the first primary NRTI-resistance mutation together with V75M in child NYU79. NYU33 developed K219Q only, a "secondary" NRTI-resistance mutation.

Emerging Pattern of NNRTI Resistance-Associated Mutations

In four of the five children who received nevirapine (NYU69, NYU70, NYU85, NYU90) K103N appeared as the first primary NNRTI-resistance mutation, while in one (NYU62) G190A appeared as the first mutation (Table III). In two of the five children who received efavirenz (NYU44 and NYU 83) K103N appeared as the first NNRTI-resistance mutation, while in two children (NYU30 and NYU33) L100I and K101Q, respectively, appeared as the first NNRTI-resistance mutation. One child (NYU36) who received didanosine/lamivudine/abacavir with subsequent change to an efavirenz-containing regimen developed I178M as the first NNRTI-resistance mutation, which was replaced later by appearance of G190A.

One child (NYU79) developed K101E and G190A as first NNRTI-resistance mutations with nevirapine therapy and developed additionally Y181C when ART was changed to efavirenz-containing regimen during the study period.

In the remaining one child (NYU38) no known NNRTI-resistance mutation was detected despite receiving nevirapine—and later efavirenz-containing regimen (Table III).

Growth of Minor Mutant Virus Population into Major One

Five of 12 children had RTI-resistance mutations detected as minor virus populations, which subsequently grew into full clones (Table IV). In the remaining seven children no RTI-resistant mutation was detected as a minor population (data not shown).

RTI-resistance mutations, such as T215F in child NYU36, T215F in NYU44, D67N/K70R/T215F in NYU62, and K101Q/K219Q in NYU33, appeared as minor populations after initiation of treatment, which overgrew subsequently to major populations.

In one child (NYU69), K103N was found as a minor population (1/5 clones) before initiation of treatment and became major population (7/7 clones) 8 months after treatment.

TABLE III. Patterns of NNRTI*-Resistance Mutations Among Non-B Subtype HIV-1-Infected Children With Treatment

Child (ID)	Study point (mpti[a])						Treatment
	Pre-treatment	1st	2nd	3rd	4th	5th	
NYU69	*K103N* (−4)	*K103N* (10)					NEVIRAPINE
NYU70	*K103N* (−10, −1)	*K103N* (11)					
NYU85		*K103N* (9)	*K103N* (12)				
NYU62		*G190A* (6)	*G190A* (12)	*G190A + Y181C* (26)			
NYU90	*Y181C* (−0.25)		*K103N* (11)				
NYU38							
NYU83		*K103N* (12)	*K103N* (22)	*K103N* (38)			
NYU30				*L100I* (31)			
NYU44	*K103N* (−3)	*K103N + G190A* (10)	*K103N+G190A* (18)				EFAVIRENZ
NYU33			*K101Q* (11)	*K101Q* (34)			
NYU36			*I178M* (13)	*G190A* (16)	*G190A* (24)	*G190A* (34)	
NYU79		*K101E + G190A* (10)	*K101E + G190A + Y181C* (14)				
NYU38							

NNRTI: non-nucleoside analogue RTI.
[a]mpti, months post treatment initiation; blank, no mutation detected.

TABLE IV. Evolution of Minor RTI-Resistance Mutant Populations Among Non-B HIV-1-Infected Children With Treatment

Child ID	Study point (months post treatment)	ART[a]	Plasma viral load (copies/ml)	NRTI[b]-resistance mutations	NNRTI[c]-resistance mutations
NYU36	1st (6)		114,754	**T215F (1/9)[d] + M184V (6/8)**	
	2nd (13)	DDI, 3TC, ABC	880,405	**T215F (1/8) + M184V (2/8)**	I178M (6/8)
	3rd (18)	ZDV, DDI, EFV	81,870	**T215F (9/9) + M184V (8/9)**	G190A (8/9)
	4th (24)		607,224	**T215F (5/5)**	G190A (5/5)
	5th (34)	D4T, DDI, EFV	393,420	**T215F (7/7)**	G190A (7/7)
NYU44	Pre-treatment		1,017,931		K103N (5/5)
	1st (10)	ZDV, DDI, EFV	71,895	D67N (5/5) + K70R (5/5) + **T215F (1/5) + K219Q (5/5)**	K103N (5/5) + G190A (5/5)
	2nd (17)		150,549	D67N (5/5) + K70R (5/5) + **T215F (5/5) + K219Q (5/5)** + **M41L (1/5)** + V75M (3/5)	K103N (5/5) + G190A (5/5)
NYU62	Pre-treatment		239,644		
	1st (6)	ZDV, 3TC, NVP	2,838	**D67N (1/5) + K70R (1/5)**	G190A(5/5)
	2nd (12)			**D67N (5/5) + K70R (5/5) + T215F (2/5)** + K219E (5/5)	G190A (5/5)
	3rd (26)		6,901	**D67N (5/5) + K70R (5/5) + T215F (2/5)** + K219E (5/5)	Y181C (4/5) + G190A (5/5)
NYU69	Pre-treatment		227,176		**K103N (1/5)**
	1st (10)	ZDV, 3TC, NVP	113,868	M184V (7/7)	**K103N (7/7)**
NYU33	1st (15)		3,449		
	2nd (23)	ZDV, 3TC, EFV	122,419	**K219Q (4/11)**	**K101Q (6/11)**
	3rd (34)	ZDV, DDI, EFV	6,457	**K219Q (14/14)** + D218E (14/14)	**K101Q (14/14)**

[a]ART, anti-retroviral therapy; ZDV, zidovudine; ddI, didanosine; EFV, efivarenz; NVP, nevirapine; 3TC, lamivudine; d4T, Stavudine.
[b]NRTI, nucleoside analogue RTI.
[c]NNRTI; non-nucleoside RTI, blank; no mutation detected.
[d]Number of clones with mutation/ total number of clones analysed; bold, minor RTI-resistant mutant populations that evolved.

DISCUSSION

In the current study, NNRTI resistance-associated primary mutations, K103N and Y181C, were found before ART in four (33.3%) of 12 HIV-1-vertically-infected Kenyan children with subsequent ART failure. Three children aged 5–7 years already had K103N mutation, while one child aged 1 year already had Y181C by the time ART was started. These children had no history of previous exposure to any ART or blood transfusion, suggesting that these drug-resistance mutations were transmitted vertically from their mothers. However, ART history of these children's mothers could not be confirmed, and the use of nevirapine to reduce transmission of HIV-1 from mother to child had not been started by the year 2002 in Kenya [NASCOP, 2002].

This is the first report on the long-term persistence of NNRTI-resistance mutation for upto 7 years in vertically HIV-1-infected children albeit in the absence of ART. The K103N mutation has been reported to have little impact on the replicative capacity of HIV-1, allowing K103N variants to persist as dominant species at the expense of the wild strains [Brenner et al., 2002]. Thus, these current findings emphasize the need for drug-resistance testing among HIV-1-infected children prior to starting any NNRTI-containing regimen to avoid earlier treatment failure.

The selection of some ARV-resistance mutations among minor HIV-1 populations after ART initiation has been reported previously [Coffin, 1995; Metzner et al., 2003; Charpentier et al., 2004; Dykes et al., 2004; Lecossier et al., 2005]. In this study, RTI-resistance mutations detected in five children as minor populations after ART initiation subsequently grew into major populations, resulting in ART failure. In addition, it is noted that a primary NNRTI-resistance mutation, K103N, was found in one of five HIV-1 clones from a drug-naïve Kenyan child (NYU69), and this minor drug-resistant virus became dominant (seven of seven clones) after 8-months ART, resulting in treatment failure. These findings indicate that minor ARV-resistant HIV-1 variants existing before therapy can also be an important cause of treatment failure, as suggested previously [Dykes et al., 2004; Lecossier et al., 2005; Johnson et al., 2006]. Standard genotyping methods can only detect more than 25% of the virus variants [Gunthard et al., 1998]. Therefore, in order to pick minor variant populations and pre-empt treatment failure, more sensitive detection methods for minor HIV-1 populations would be required [Edelstein et al., 1998; Gunthard et al., 1998; Grant et al., 2002; Schuurman et al., 2002; Malet et al., 2003; Shi et al., 2004; Palmer et al., 2005].

Results from this study suggest the possible existence of two different patterns of emergence or acquisition of the TAMs among children who receive thymidine-analogues such as zidovudine, lamivudine, and/or stavudine. Seven of the 12 children had an initial development of M184V mutation, followed by the cumulative acquisition of TAMs, consistent with previous studies of subtype

B HIV-1 [Johnson et al., 2005], which reported that TAMs always develop by either one of two distinct pathways, TAM1 (M41L, L210W, and T215Y) or TAM 2 (D67N, K70R, and K219Q/E/R), under the pressure of thymidine analogue-containing ARVs. The remaining five children, however, developed TAMs only without the initial appearance of M184V mutation. Additionally, three of these children developed both TAMs 1 and 2 members concurrently, discordant with previous reports [Flandre et al., 2003; Cozzi-Lepri et al., 2005]. One child (NYU33) developed K219Q and K101Q mutations only, after 2-year treatment with zidovudine, didanosine, and efavirenz. These two mutations have been previously grouped among the secondary RTI-resistance-associated mutations, unable to cause drug-resistance in the absence of other primary RTI-resistance-associated mutations such as K70R or T215F [Garcia-Lerma, 2005]. These findings therefore suggest the possible existence of different pathways for development of RTI-resistance in non-subtype B HIV-1-infected children, different from those reported in subtype B-infected individuals, and that secondary RTI-resistance-associated mutations namely K219Q and K101Q could independently cause ART resistance among non-subtype B HIV-1-infected children. Further studies are however needed in order to confirm these findings.

The K103N mutation has been reported as the most commonly selected NNRTI-resistance-associated mutation, usually appearing first [Johnson et al., 2005]. The results from the children who received nevirapine in this study agree with this observation. However, the children who received efavirenz developed a variety of NNRTI-resistance-associated mutations, such as L100I, K101Q, I178M, and G190A. This is the first report to show the possibility of the K101Q and I178M to appear as the first NNRTI-resistance mutations with efavirenz therapy. L100I, Y181C, and G190A have already been described [Johnson et al., 2005]. In addition, one child (NYU38) who received nevirapine and later efavirenz containing regimen did not have any NNRTI-resistance-associated mutation despite experiencing treatment failure, suggesting a possible difference in the initial selection of NNRTI-resistant mutations between non-subtype B and subtype B HIV-1-Infected children. However, considering recent reports on the association between a homozygous variant of multidrug-resistance transporter *C3435T* and good immune recovery [Saitoh et al., 2005], and the correlation of homozygous *CYP2B6* *6 with plasma efavirenz concentrations in HIV-1-infected individuals treated with efavirenz-containing regimen [Tsuchiya et al., 2004], further pharmacogenetic studies would also be needed to elucidate these phenomenon.

In conclusion, this study suggests a possible long-term persistence of "vertically transmitted" NNRTI-resistance mutations in the absence of drug pressure, that minor populations of RTI-resistant HIV-1 mutants may impact negatively on the outcome of ART, and that there is a possible difference in the pattern of appearance and profile of RTI-resistance mutations between non-subtype B and subtype B HIV-1-infected children. Further studies with large population size are needed to confirm these findings.

SEQUENCE DATA

GenBank accession numbers of the sequences reported in this study are DQ679541 to DQ679753 for *Pol-RT*.

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Feature Article

Factors determining prenatal HIV testing for prevention of mother to child transmission in Dar Es Salaam, Tanzania

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Abstract

Background: The objectives of the study were (i) to evaluate the Prevention of Mother to Child Transmission (PMTCT) services in Temeke district, Tanzania and (ii) to identify factors for non-acceptance of HIV testing among pregnant mothers in the area.

Methods: A structured questionnaire was used in face-to-face interviews at five health centers in the district. Univariate and multiple logistic regression analyses were used to assess the association of the refusal of human immunodeficiency virus (HIV) testing with risk factors.

Results: Two hundred and seventy-three (68.1%) of the participants had already had HIV testing, while 128 (31.9%) had not. Participants' general knowledge of HIV was high, but specific knowledge of mother to child transmission (MTCT) was relatively low. In the multiple logistic regression analysis, frequencies of antenatal clinic visits, awareness of MTCT and intensive family support were significantly and inversely associated with the refusal of HIV testing.

Conclusions: Frequency of antenatal care visits, spreading information on HIV/acquired immune deficiency syndrome especially MTCT, and husbands' intensive support are significant factors for increase of HIV test acceptance among pregnant women in the study area.

Key words HIV testing, mother to child transmission, pregnant women, Preventing Mother to Child Transmission program, Tanzania.

Mother to child transmission (MTCT) of human immunodeficiency virus (HIV) is the major source of HIV infection in children. There are 40.3 million people infected with HIV globally and 2.3 million are children under 15 years of age. Sub-Saharan Africa remains hardest hit, with 25.8 million people living with HIV. Two-thirds of all people living with HIV are in Sub-Saharan Africa, as are 77% of all women with HIV.[1] In Tanzania, the first acquired immune deficiency syndrome (AIDS) cases were diagnosed in 1983.[2] During the 1990s the prevalence of HIV in Tanzania fluctuated greatly. In 2000 it was reported that approximately 1 800 000 people aged ≥15 were living with HIV. Of them, >900 000 women were of reproductive age (15–49 years).[3] The main mode of transmission is through heterosexual intercourse, accounting for 78% in 2001, and MTCT ranked second, contributing up to 5%.[2]

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In Tanzania, the estimated number of women who become pregnant per year is 1.3 million and HIV prevalence among women attending antenatal clinics in 2002 was 9.6%.[2,4,5] In addition, data from the Tanzania Ministry of Health (MOH) show that the risk of transmission from HIV-infected pregnant women to their newborns is 40%, distributed as follows: 10% *in utero*, 20% during labor and delivery, and 10% through breast-feeding.[4] The Preventing Mother to Child Transmission (PMTCT) of HIV program aims at reducing MTCT of HIV during pregnancy, childbirth and during breast-feeding. It includes voluntary counseling and testing (VCT), antiretroviral (ARV) treatment and counseling about feeding options (Fig. 1). In 1998, after finding that a short course of the antiretroviral drug Zidovudine (ZDV; GlaxoSmithKline, Middlesex, UK) starting from 36 weeks of pregnancy reduced the rate of MTCT by 50%, a comprehensive PMTCT strategy was developed. The PMTCT project in Tanzania was launched in 1999, and started operation from five centers. In 2002, an evaluation report for the project showed some weaknesses and constraints in PMTCT services.[6,7] One of the major barriers was its low

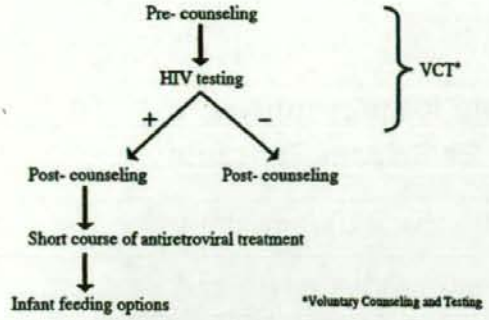

Fig. 1 Preventing Mother to Child Transmission.

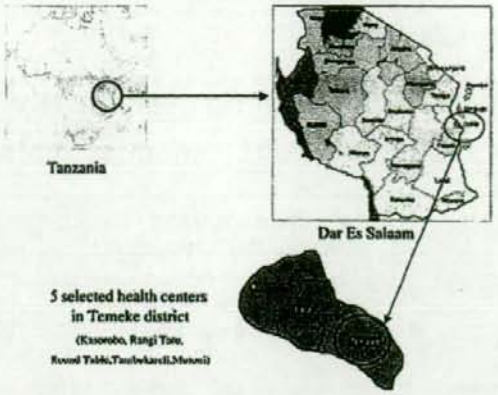

Fig. 2 Map of study site.

acceptance. The report also showed large differences in counseling rates (9–56%), high acceptance of rapid HIV testing among those who received counseling (78–84%), and low short-course ZDV uptake (8–20%).

Temeke district was chosen for the following three reasons. First, the Dar Es Salaam region has the highest HIV/AIDS case rate (235 per 100 000) compared to other regions.[8] A previous survey showed that prevalence of pregnant women varies by geographical settings, with much higher levels in border towns, along major highways and in urban centers as compared to rural areas.[2] Second, in the Dar Es Salaam region, the Temeke district represents a typical urban city because the other districts are a center for government and commercial offices and a residential area for foreign and upper classes. Third, although coverage of PMTCT is very limited elsewhere in the country, the project had been extended to the health center level in Temeke.

No study has been done in Tanzania despite that acceptance of the program has been less than optimal. Therefore, the aim of the present study was to determine the reasons for low acceptance of PMTCT and to recommend proper measures to be taken in Tanzania. The objectives of the study were (i) to evaluate the PMTCT services that are currently provided and the knowledge and attitude on counseling and testing of HIV among pregnant mothers in Temeke district, Tanzania and (ii) to identify factors for non-acceptance of counseling and testing for HIV among pregnant mothers in the area.

Methods

Study area

The study was conducted at five health centers in Kasorobo, Mtoni, Rangi Tatu, Round Table and Tambukareli in Temeke district, Dar Es Salaam, Tanzania (Fig. 2). Tanzania has a total area of 940 000 km^2, and is divided into 26 administrative regions and 131 districts. The total population is estimated at approximately 34.5 million and the population growth rate is 2.9% per year.[9] Although Dar Es Salaam is not a capital anymore, it is still the center of politics and economy. Temeke district (municipality) is in the southern part of the Dar Es Salaam Region. The region consists of three districts and Temeke is the largest one. It has an estimated population of 768 451 and the population growth rate is 7.8% (Temeke municipal council, unpubl. obs., 2004).

Study population

The target population consisted of pregnant women attending antenatal care at the selected health centers where PMTCT programs are implemented. Because the rates of access to antenatal care services were 98% (one visit) and 70% (four times or more) in Tanzania, random sampling was applicable in the present study.[10] Then the sample size was calculated from the previous records in the area using EpiInfo version 6 (Centers for Disease Control and Prevention, Atlanta, USA) and 401 pregnant women were enrolled.

Study design and procedure

This was an exploratory and cross-sectional study, conducted from August to September 2004. A structured questionnaire was used in face-to-face interviews with pregnant women at the health centers by a trained interviewer in the Swahili language. All women seeking antenatal care were approached individually and asked to participate in the study. Verbal consent was obtained from all participants. Each interview was conducted in an isolated space and lasted approximately 15–20 min. Incentives were not provided for participation. To preserve confidentiality and because of concerns about unnecessary disclosure, the participants' actual HIV status was not

collected. Permission was obtained from Tanzania Commission for Science and Technology, Ministry of Health in Tanzania and the Ethical Review Committee, Graduate School of Medicine and Faculty of Medicine, University of Tokyo.

Questionnaire

In order to identify factors associated with refusal of HIV testing among pregnant women, a structured questionnaire was designed. The dependent variable was 'not being tested for HIV'. Demographic factors (age, educational background, religion, marital status, family size and number of children), gestational age, frequencies of health center and antenatal visits, travel time from home to health centers, general knowledge of HIV, specific knowledge of MTCT of HIV, and attitude toward HIV testing were introduced as independent variables.[11]

For measuring attitude toward HIV testing, the modified HIV-Antibody Testing Attitude Scale developed by Boshamer and Bruce in 1999 was added to the questionnaire.[12] It was used to assess respondent concern about family support, social support, and privacy. Scores on the scales were positively correlated with perceived knowledge about HIV and the likelihood of being tested.[12]

Statistical analysis

Statistical analyses were performed using the SPSS 11.0J program (SPSS Japan, Tokyo, Japan). Univariate and multiple logistic regression analyses were used to assess the association of the refusal of HIV testing with each risk factor. The χ^2 test was used to compare the rates in different categories. Variables that had a significant relationship to the dependent variable at the $P < 0.05$ level were then used in the multiple logistic regression analysis and selected by using backward stepwise procedure.

Results

Characteristics of respondents and acceptance rate of HIV testing

Four hundred and one pregnant women were interviewed at five health centers in the Temeke district. The participants characteristics are given in Table 1. Two hundred and seventy-three (68.1%) had already had HIV testing, while 128 (31.9%) had not. The acceptance rate of testing in the present study was larger than that in 2003 (42.6%). Among the five health centers, no statistical difference was seen in those who had been tested and those who had not. None of the social demographic factors was significantly associated with an acceptance of the HIV testing.

Table 1 Participant characteristics ($n = 401$)

Variables	Mean	SD	*n*	%
Domicile				
Kasorobo			48	12.0
Mtoni			49	12.2
Rangi Tatu			120	30.0
Round Table			121	30.2
Tambukareli			63	15.7
Age (years)	24.18	5.53		
Gestational age (months)	6.84	1.85		
Education (years)				
≦7			362	90.3
>7			39	9.6
Marital status				
Married			323	80.5
Single			29	7.2
Others			49	12.1
Family size	4.34	2.51		
No. children	1.17	1.39		
Distance to health centers (min)	26.03	24.96		
Transportation				
On foot			290	72.6
By public bus			105	26.2
Others			6	1.5
Travel time (min)	30(mode)			
No. health center visits	2.01	1.34		
No. antenatal care visits	2.67	1.69		
HIV testing				
Tested			273	68.1
Not tested			128	31.9

Among those previously tested, 69.2% of participants ($n = 189$) answered that the final decision was made by themselves, and 52.3% ($n = 67$) of those who had not been tested responded that they would like to be tested in the future. Major reasons of refusing test were the following: 'I don't have enough information about testing' (58.4%), 'I don't know the benefits of knowing my HIV status' (40.4%), 'I can't get any medication if I am found HIV positive' (32.6%) and 'I'm afraid of knowing my HIV status' (31.5%). In contrast, the major reasons for acceptance of testing were the following: 'I'm interested in knowing the results' (98.2%), 'It's beneficial to me' (94.9%), 'It's beneficial to my baby' (94.9%), 'I had enough information about testing' (94.1%) and 'I'm not afraid of knowing the status' (93.4%).

Awareness of HIV

Participant awareness of HIV is shown in Figure 3. A total of 98.2% of those who had been tested for HIV and 98.4% of

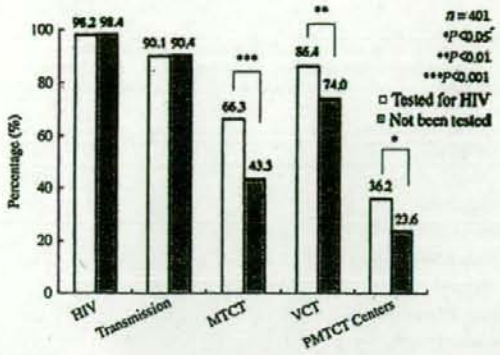

Fig. 3 Awareness of HIV. MTCT, mother to child transmission; PMTCT, prevention of mother to child transmission; VCT, voluntary counseling and testing.

those who had not been tested had heard of HIV/AIDS. A total of 90.1% of those who had been tested and 90.4% of those who had not understood how the disease is spread (main answers: sexual intercourse and sharing sharp edges). In contrast, the awareness of MTCT of HIV was relatively low: 66.3% of those who had been tested and 43.3% of those who had not, answered correctly. Sources of information about MTCT of HIV are given in Figure 4. Among those who were aware of MTCT of HIV, 68.4% answered that they learned about it from antenatal care visits, 43.7% from radio, and 26.9% from friends and neighbors.

Knowledge of MTCT of HIV

The knowledge of MTCT was also low, as shown on Table 2. The percentage of correct responses to the statement that HIV-positive pregnant women can transmit the virus to their babies during pregnancy was 49.4% of those who had been tested for HIV and 45.4% of those who had not been tested; for transmission during delivery it was 55.7% of those who had been tested and 50.0% of those who had not; and for during breast-feeding it was 63.5% of those who had been tested and 60.0% of those who had not, respectively. Moreover, 23.8% of those who had been tested and 20.3% of those who had not correctly answered that HIV-positive mothers always deliver HIV-positive babies. 38.7% of those who had been tested and 22.3% of those who had not knew of medicine for PMTCT of HIV, and statistical significance was found between these two groups.

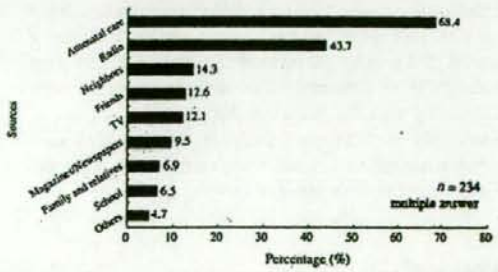

Fig. 4 Sources of information about mother to child transmission of HIV.

Table 2 Knowledge of MTCT of HIV

Queries	Percentage of correct answers	
	Tested for HIV ($n = 273$)	Not tested for HIV ($n = 128$)
MTCT during pregnancy?	49.4	45.4
MTCT during delivery?	55.7	50.0
MTCT during breast-feeding?	63.5	60.0
If a baby tests positive means a mother positive?	78.2	70.8
HIV-positive mother always deliver HIV baby?	23.8	20.3
Medicare for MTCT?	38.7	22.3***

***$P < 0.001$. MTCT, mother to child transmission.

Attitude toward HIV testing

The result of the attitude scale is shown in Table 3. It used a three-point Likert scale: agree, neutral, disagree. The mean score of family support (items: 'My family would support me if I decided to be tested for HIV', 'I could easily discuss HIV testing with my family' etc.) was 11.03 ± 3.204 (range: 9–27). On this scale, 9 was the strongest and 27 was the weakest evidence for family support. The mean score of the social support (items: 'I would not want anyone to know if I got an HIV test', 'People would assume I have HIV if I decided to get tested' etc.) was 12.15 ± 3.136 (range: 9–25). On this scale, 9 was the strongest and 25 was the weakest evidence for community support. The mean score of privacy (items: 'I am afraid that if I were to be tested for HIV, my name would go onto public records', 'HIV testing information is kept very confidential by the medical staff who do the testing' etc.) was 9.06 ± 2.414 (range: 7–21). On this scale, 7 was the strongest and 21 was the weakest evidence for protection of their privacy.

Table 3 Attitude scale toward HIV testing

	Range	Mean	SD
Family support	9–27	11.03	3.20
Social support	9–25	12.20	3.14
Privacy	7–21	9.06	2.41

Univariate analysis

Univariate analysis was used to assess the association of the refusal of HIV testing with potential risk factors. The result is shown in Table 4. Participants who were aware of MTCT of HIV, VCT and PMTCT centers, and the lower score of family support, social support and privacy were significantly inversely associated with refusal of HIV testing. Concerning knowledge of medication of PMTCT, the result did not reach statistical significance.

Multiple logistic regression analysis

Multiple logistic regression analysis was used to assess the association of non-acceptance of HIV testing with each risk factor. The following variables were used for selection of independent variables: (i) gestational age; (ii) number of health center visits; (iii) number of antenatal clinic visits; (iv) frequencies of antenatal clinic visit; (v) awareness of MTCT; (vi) awareness of VCT; (vii) awareness of PMTCT cites; (viii) knowledge of MTCT of HIV; (ix) family support; (x) social support; and (xi) privacy.

Table 4 Univariate analysis of HIV testing refusal

Variables	Crude OR	95%CI
No. antenatal clinic visits		
≥2	0.152	0.095–0.245
<2	1	
Awareness of MTCT of HIV		
No	2.575	1.671–3.968
Yes	1	
Awareness of VCT		
No	2.230	1.317–3.776
Yes	1	
Awareness of PMTCT centers		
No	1.834	1.136–2.962
Yes	1	
Knowledge of medication of PMTCT		
No/Don't know	1.132	1.004–1.277
Yes	1	
Score of family support		
>11	1.360	1.245–1.485
≤11	1	
Score of social support		
>12	1.156	1.081–1.236
≤12	1	
Score of privacy		
>9	1.277	1.166–1.399
≤9	1	

CI, confidence interval; MTCT, mother to child transmission; OR, odds ratio; PMTCT, prevention of mother to child transmission; VCT, voluntary counseling and testing.

The independent variables were selected using a stepwise procedure. Three independent variables (frequencies of antenatal clinic visits, intensive family support and awareness of MTCT) were used in the final model (Table 5). These were independently, significantly and inversely associated with refusal of HIV testing.

Discussion

Many pregnant women were willing to take the HIV test, but there were some constraints. Although previous studies noted that the demographic characteristics were associated with acceptance of HIV testing, none of that was statistically significant in the present study.[13–15] More frequent antenatal visits by the respondents was significantly inversely associated with non-acceptance of the testing in both univariate and multivariate analysis. Late or no prenatal care were variables independently associated with a lower probability of being HIV tested.[16] Beginning antenatal care early in pregnancy and frequency of health care and antenatal care visits might influence the increase of the test acceptance because the PMTCT program is carried out mainly by nurses or counselors.

As shown in the results, general knowledge of HIV was well-spread in the study area, but prevalence of specific knowledge of MTCT remained problematic. The results showed that the acceptance of HIV testing might increase when women understand MTCT of HIV and the role of medication, which is in agreement with previous studies.[14,17,18] Information and greater knowledge of MTCT of HIV are crucial in improving acceptance of the testing. The low levels of the knowledge can be explained by a lack of necessary information. Radio, friends and relatives as well as antenatal care were the main sources for knowledge of HIV/AIDS in the present study, which was consistent with previous reports.[2,19,20] Because the educational levels of respondents in the study were relatively low, the dissemination of information on HIV/AIDS may be effective

Table 5 Multiple logistic regression analysis of HIV testing refusal

Selected variables	Adjusted OR	95%CI
No. antenatal clinic visits		
≥2	0.571	0.450–0.724
<2	1	
Awareness of MTCT of HIV		
No	2.423	1.299–4.520
Yes	1	
Score of family support		
>11	1.358	1.231–1.498
≤11	1	

CI, confidence interval; MTCT, mother to child transmission; OR, odds ratio.

through radio and TV. Radio can be made to provide two-way communication, giving women the chance to talk about some of their concerns.

Support from husbands and families was also an important factor in deciding to be tested. Traditionally, in Tanzanian society, women cannot voice their individual opinions.[21] As shown in Figure 5, 60.9% of women answered that their husbands were the key persons to decide important domestic issues, while 25.5% of women answered that household decisions were made mutually. It suggested that the power and gender relations in this cultural setting are generally in favor of men, and that women tend to conceal matters that may put their relationships with men in jeopardy.[22] Moreover, they are fearful of violence, divorce, and of being left alone to die if they are found to be infected with HIV.[7,23–28] Women need to consult their husbands before getting tested, which is one of the reasons for refusing HIV testing. Although 70.0% of those who were tested answered that they decided by themselves, the PMTCT program should be extended to husband or partners.

In the univariate analysis, lack of community support, privacy and confidentiality of counselors were responsible for the low acceptance of testing. Pregnant women were afraid of being stigmatized or discriminated against in the community as well as being shunned by family.[7,17,29] Bunting noted that the main causes of this fear were related to insufficient and inaccurate knowledge, fears of death and disease, sexual norms and a lack of recognition of stigma.[30] Knowledge of HIV/AIDS and MTCT of HIV should also be expanded to the community for reducing ignorance and prejudice, and HIV counseling plays an important role in reducing women's fear of and stigma associated with the testing.[31,32]

The present study has several limitations. First, the study was done in only the urban area. In Tanzania as well as other developing countries, rural living conditions are much more difficult than that of urban areas. Stigma and discrimination leading to denial of HIV may be deep-rooted, and agreeing to HIV testing may be more stressful to women living in the local setting. Further research will be needed, especially in rural areas, in order to grasp the whole situation in Tanzania. Second, the study needs to focus more on the counseling aspect of the PMTCT program. Health-care providers play an important role in increasing acceptance of the program and in maintaining participant privacy. Many counselors' training courses were held under the auspices of the MOH and other organizations including non-government organizations in the region. However, the qualities of HIV counseling in the study area are still questionable. Considering these situations, further studies are needed for the successful expansion of the PMTCT program and to ensure the best possible outcome for both HIV-positive pregnant women and their children.

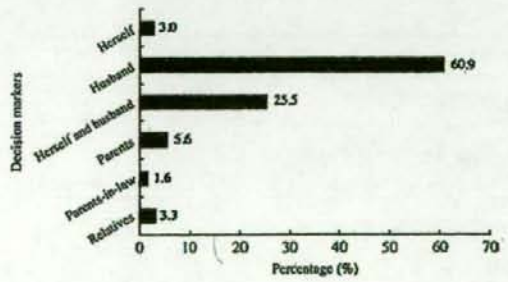

Fig. 5 Final decision on domestic issues.

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Possible Misidentification of GSP[6] Rotavirus as a Novel Strain Detected in Humans for the First Time

Acute gastroenteritis has been demonstrated to be a major cause of morbidity and mortality of children in both developed and developing countries (12, 13). It has been well established that virtually every child has become infected with a rotavirus at least once by 3 years of age (15, 17). The rotaviruses, which comprise a genus in the family *Reoviridae*, are spherical in appearance and measure about 70 nm in diameter. Rotaviruses contain 11 segments of double-stranded RNA. Rotaviruses are classified into seven groups (A to G) on the basis of their distinct antigenic and genetic properties. Human infection has been reported with group A, B, and C rotaviruses (4). Of these, group A rotavirus is the most important, being a significant cause of severe gastroenteritis in children worldwide (13, 15). The two outer capsid proteins, VP7 and VP4, allow the classification of rotaviruses into G and P genotypes, respectively. In rotavirus, at least 16 G and 27 P genotypes have been recognized (4, 7, 8). Of these, five rotavirus G-P combinations, G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8], are the most common globally and are therefore the targets for current vaccine development strategies (16).

Recently Duan et al. identified a rotavirus strain, LL36755, in the fecal specimen collected from a female patient with acute gastroenteritis in China in 2003 by reverse transcription-PCR (3). By BLAST analysis, strain LL36755 shared high identities, from 92% to 95%, in the amino acid sequences of VP7 and VP4 to those of the other G5 and P[6] rotavirus reference strains in GenBank. Rotavirus strains sharing >89% VP7 or VP4 amino acid sequence identity are considered to belong to the same G or P genotype, respectively (3, 5, 6). Obviously, strain LL36755 qualified as a G5P[6] rotavirus (3). According to this research group, strain LL36755 represented the first identification of a human rotavirus G5P[6] combination in the world and was proposed to be the novel rotavirus strain (3). However, it was found that an unusual G5P[6] combination had already been detected in a single case of reinfection among children participating in a trial with rhesus-human reassortant tetravalent vaccine in Belem, Brazil, from 1990 to 1992, in a previously published study (11). Like strain LL36755, this unusual rotavirus strain from Brazil also demonstrated a long RNA pattern in polyacrylamide gel electrophoresis (11). Taking the data together, the results clearly indicated that strain LL36755 was not a novel strain or the first human rotavirus G5P[6] strain as Duan et al. stated in the recent publication (3). G5 and P[6] rotaviruses were originally isolated from pigs, and then many G5 and P[6] rotaviruses were found in humans (1–3, 9, 10, 14, 16). There might exist a genomic relatedness between human and porcine rotaviruses, and porcine rotaviruses were regarded as a potential reservoir for genetic/antigenic diversity of human rotaviruses (3, 10, 16). Therefore, observation of close homology of VP7 and VP4 genes between strain LL36755 and porcine rotavirus in the recent publication of Duan et al. was not surprising.

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Author's Reply

I thank Phan et al. for pointing out the previous description of a G5P[6] rotavirus in a single case of reinfection among children participating in a trial with rhesus-human reassortant tetravalent vaccine in Belem, Brazil, from 1990 to 1992, in a report published in 2002 (2). I sincerely apologize for the inadvertent omission of this Brazilian study in our case report. We had not read this paper at the time of submission of our manuscript due to limited circulation of the journal in China. Unfortunately, we had no access to either an electronic version or a print version of the full paper, while the description of the G5P[6] strain in the abstract was ambiguous.

Upon careful review of the Brazilian study, we found that the reported detection of the G5P[6] strain was based on PCR and hybridization. No nucleotide sequence information on that G5P[6] strain was determined in the study or deposited in the GenBank database. Thus, in our view, the data presented in the Brazilian study were insufficient in demonstrating the existence of a novel G5P[6] rotavirus at the nucleotide sequence level.

In this connection, our case report represents the first detection of a human G5 rotavirus in Asia and the first verification of the unusual combination of G5 and P[6] genotypes in humans (1). The nucleotide sequence information reported in our work provides a very critical piece of data in genotype analysis of this rotavirus and in the study of its origin. In this sense, our work does verify at the nucleotide sequence level that this is indeed a novel human rotavirus of the G5P[6] genotype.

I agree with Phan et al. that the striking sequence homology between the LL36755 strain and porcine rotaviruses is not surprising. However, our sequence data do implicate that interspecies transmission has occurred. Consistent with the importance of interspecies transmission, our continuing study has confirmed multiple sporadic cases of human infection with G5P[6] rotavirus in the local area.

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Short communication

Emergence of intragenotype recombinant sapovirus in Japan

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Abstract

Sapovirus is an important causative agent of sporadic cases as well as of outbreaks of acute gastroenteritis in humans worldwide. A total of 603 fecal specimens collected from July 2005 to June 2006 from children with acute gastroenteritis in five localities in Japan (Maizuru, Tokyo, Sapporo, Saga, and Osaka) were screened for sapovirus by RT-PCR. It was found that 17 specimens were positive for sapovirus and it represented 2.8%. Interestingly, intragenotype recombinant sapovirus GI/1 emerged with 76.4% (13 of 17) and rapidly became the leading cause of acute gastroenteritis in Japan for the first time. The lower frequency of sapovirus GI/2 and GI/4 (each of 11.8%), which were the second prevailing genotypes, was also detected. A novel nomenclature of sapovirus was proposed, in which worldwide sapovirus strains were classified into seven genogroups. Of these, novel sapovirus genogroups VI and VII demonstrated the very low homologies, only 32.8–41.6% at the amino acid level and 43.6–49.9% at the nucleotide level, to those of sapovirus genogroups I–V. Of note, two distinct clusters of sapovirus were co-circulating in porcine. Interestingly, the worldwide sapovirus strains shared the 25 nucleotide-conserved region, covering the polymerase–capsid junction which differed according to each species due to multiple nucleotide substitutions. The finding suggests that the sapovirus recombination between human and animal hardly takes place in nature. This is also the first, to our best knowledge, demonstrating the emergence of the intragenotype recombinant sapovirus with its causing diarrheal illness in Japan.

Keywords: Sapovirus; Emergence; Genogroup; Recombination; Japan

1. The study

Viral gastroenteritis is a common disease with a high morbidity reported worldwide, especially in infants and the elderly. The mortality in children due to gastroenteritis is greater in developing than in developed countries. Acute gastroenteritis ranks consistently as one of the principal six causes of all deaths (Murray and Lopez, 1997; Parashar et al., 2003; Thapar and Sanderson, 2004). Sapovirus is recognized as a significant global enteropathogen, being a common cause of sporadic cases as well as of outbreaks of acute nonbacterial gastroenteritis in humans of all age in various epidemiological settings such as kindergartens, schools, and nursing home for the elderly (Chiba et al., 1979, 2000; Lopman et al., 2002; Akihara et al., 2005; Yan et al., 2005). Sapovirus is the distinct genus within the family Caliciviridae. The sapovirus genome contains two ORFs. The ORF1 encodes non-structural and capsid proteins while ORF2 encodes a small protein. Sapovirus has a typical "Star of David" configuration by electron microscopy. The prototype sapovirus is the Sapporo virus (Hu/SaV/Sapporo virus/1977/JP), which was originally discovered from an outbreak in a home for infants in Sapporo, Japan, in 1977 (Chiba et al., 1979).

A total of 603 fecal specimens were collected from sporadic cases of acute gastroenteritis in pediatric clinics, encompassing five localities (Maizuru, Tokyo, Sapporo, Saga, and Osaka) in Japan from July 2005 to June 2006. The ages of the subjects ranged from 2 months to 15 years, with a median of 26 months. The fecal specimens were diluted with distilled water to 10% suspensions, and clarified by centrifugation at 10,000 × *g* for 10 min. The supernatants were collected and the viral genomes were extracted by using a QIAamp Viral RNA kit (QIAGEN®,

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Hilden, Germany). Using PCR with specific primers SLV5317 and SLV5749 as previously reported resulted in the identification of sapovirus (Phan et al., 2005). The polymerase region was also amplified to detect recombinant sapovirus using primers SR80 and JV33. Products were sequenced directly on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using CLUSTAL X (Version 1.6). A phylogenetic tree with 100 bootstrap resamples of the nucleotide alignment datasets was generated using the neighbor-joining method with CLUSTAL X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). SimPlot software (Version 1.3) was used to compare recombinant sapovirus sequences. Reference sapovirus strains and accession numbers used in this study were as follows: Cowden (AF182760), Manchester (X86560), Hou7-1181 (AF435814), Mex11859/99 (AY157857), Sapporo/82 (U65427), Plymouth (X86559), Dresden (AY694184), Houston/90 (U95644), 6728/05/Maizuru/JP (DQ395300), Bristol/98 (AJ249939), London/92 (U956445), Parkville (U73124), MEC151A (AY144337), C12 (AY603425), SK15 (AY646855), SW278 (DQ125333), 5863/Osaka/JP (DQ401095), NK24 (AY646856), JJ681 (AY974192), LL14 (AY425671), MM280 (AY823308), QW270 (AY826426), Lyon/598 (AJ271056) and JJ259 (AY826423).

Here sapovirus was detected in 17 out of 603 specimens tested, accounting for 2.8%. Fig. 1 reveals that sapovirus was divided into three distinct genotypes 1, 2, and 4 within genogroup I (GI). Of these, GI/1 was the most predominant genotype with 76.4% (13 of 17), followed by GI/2 and GI/4 with 11.8% of each (2 of 17). Thus, there was the changing epidemiology of sapovirus genotypes in Japan with the emergence of sapovirus GI/1 together with the sudden disappearance of predominant sapovirus GI/6 in the previous year (Phan et al., 2007). All sapovirus GI/1 isolates had great homologies (99–100%) each other. Obviously, they came from the same source of infection and very likely represented the same strain, the JP-6732. By BLAST, both capsid and polymerase sequences of the JP-6732 were highly identical (99–100%) to those of the novel intragenotype recombinant sapovirus 6728/Maizuru/JP (the GI/1b polymerase and the GI/1a capsid) (Phan et al., 2006a). Taken together, the results indicated that the JP-6732 was also recognized as a recombinant strain. In contrast, all sapoviruses belonging to the GI/2 and the GI/4, the genotypes remained the same no matter the polymerase or capsid regions were analyzed.

The novel intragenotype recombinant sapovirus was first noted in a 10-month old male child with acute gastroenteritis in Maizuru City in 2005 (Phan et al., 2006a) and no additional case was reported so far. Interestingly, this virus emerged and rapidly became the leading cause of acute gastroenteritis in Japan for the first time in this study. Sapovirus capsid contains the determinants which are important for the immune recognition (Chen et al., 2004, 2006). The emergence of recombinant virus with GI/1 capsid could be explained by the insufficient antibody protection from acquired viral immunity against sapovirus GI/1 due to the lack of a trigger of the previous sapovirus GI/1 infection in the previous year.

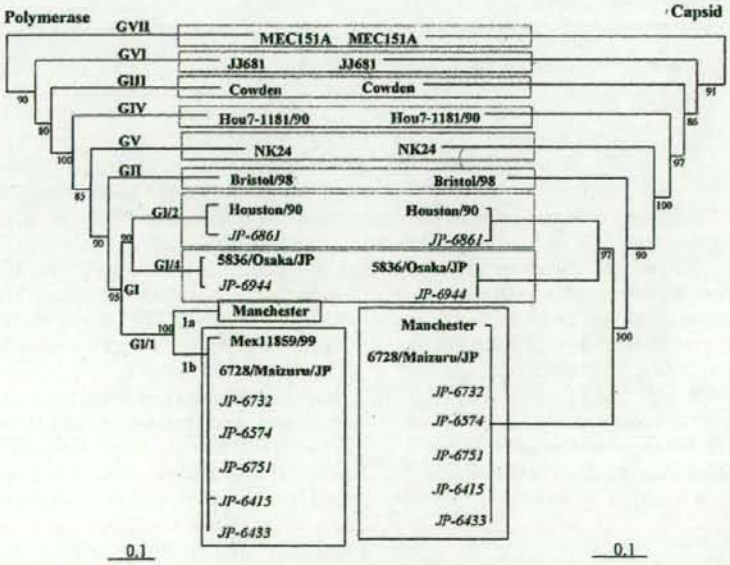

Fig. 1. Observation of changes of sapovirus subgenotypes (GI/1a and GI/1b) on the basis of phylogentic trees. The trees were constructed from nucleotide sequences of the capsid and polymerase regions of sapovirus isolates and reference sapovirus strains available in GenBank. The sapovirus isolates detected in the study are highlighted in italics. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. The novel genogroup VI (known as the JJ681 virus cluster) and novel genogroup VII (known as the MEC151A virus cluster) were also shown.

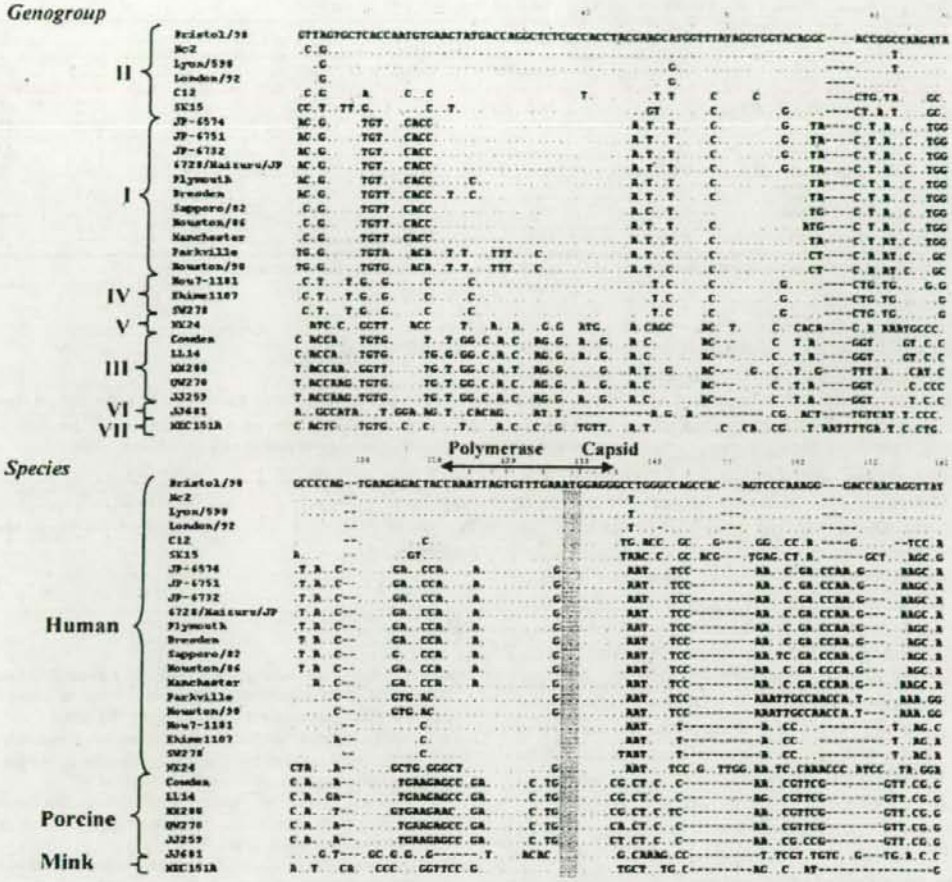

Fig. 2. Nucleotide alignment of worldwide sapovirus strains available in GenBank, showing the highly conserved region, covering polymerase and capsid junction which is indicated by a horizontal narrow. The dots represent conserved nucleotides. The shaded nucleotides represent the putative capsid start codons.