Research Article

High diversity and transmission dynamics of HIV-1 non-C subtypes in Bangladesh

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Background. Genetic diversity and molecular epidemiology of HIV are directly relevant to HIV transmission. We report here the genetic diversity and transmission dynamics of non-C subtypes of HIV-1 strains detected in Bangladeshi key populations.

Results. Sequence analysis of *gag* gene revealed four subtypes A1, B, D, G and nine CRFs (01_AE, 02_AG, 09_cpx, 10_CD, 15_AE/B, 13_cpx, 14_BG, 22_01_A1 and 25_AGU). Most of these non-C strains were detected in returnee migrant workers from different parts of the world. Phylogenetic analysis showed that the Bangladeshi HIV-1 strains detected in migrant workers and their wives and local sex workers shared common ancestries.

Conclusions. Identification of the multiple subtypes indicates high diversity of non-C HIV-1 variants circulating in Bangladesh which might have been imported by migrant workers from multiple geographical areas.

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Background

Human immunodeficiency virus 1 (HIV-1) demonstrates distinct genetic subtypes which are of major concern for vaccine development, sensitivity to antiretroviral drugs, and transmission efficiency[1]. Subtypes A, C, and D including Circulating Recombinant Forms (CRFs) are common in Sub-Saharan Africa and Asia, while subtype B is the most prevalent in the Americas. Recombination is a hallmark of HIV which generates tremendous genetic diversity among the virus population. At least 132 CRFs along with unique recombinant forms (URFs) have been reported to date (HIV Sequence database; https://www.hiv.lanl.gov/content/sequence/HIV/CRFs/crfs.comp). According to reports, 25–40% of new HIV infections in various Asian countries are caused by the wives and girlfriends of males who acquired the virus through paying women for sex, engaging in sexual activity with other men, or injecting drugs with non-sterile needles or syringes[2]. In addition, in many Asian countries, HIV has been detected among returnee migrants who acquire the infection while abroad and then transmit the virus to the local population[3][4]. A survey shows that many Bangladeshi migrant workers while abroad engage in behaviors that put them at risk of HIV[5]. Many married men claimed to have engaged in sexual activity with female sex workers, whether they had traveled within Bangladesh or abroad[6]. Compared to women who were not separated, more women whose husbands had departed reported having concomitant extramarital sex[6]. The data on the HIV epidemic relies on the national HIV surveillance that is being conducted since 1998 among key populations (KP) such as people who inject drugs (PWID), sex workers, males having sex with males (MSM), and transgendered people (referred to as hijra in Bangladesh). Additional information is gathered from the Voluntary Counseling and Testing (VCT) centers located in different parts of the country which are compiled annually by the Health Ministry of the Govt. of Bangladesh. HIV prevalence is very low in Bangladesh compared to neighboring countries (India, Nepal, Myanmar[7]), and information on HIV-1 genotypes and their possible origin and transmission is limited. In our previous genotyping report with 198 HIV-1 positive strains, we showed that genotype C and associated recombinants (CRF07_BC and CRF08_BC) accounted for 68.2 % of the HIV infections during 1999-2005 and were mainly restricted to PWID[8]. In contrast, non-C HIV-1 strains (31.8 %) were circulating in different KPs as well as in the general population. In the present study, we aimed to characterize the *gag* gene (and some *env* genes) of non-C HIV strains and investigate their origins and transmission by phylogenetic analysis.

Results

A total of 63 HIV strains were identified as non-C subtypes, 52 of which were from clients of the VCT unit and 11 from participants in the national HIV surveillance. The mean age of the study participants was 30.7 years which ranged from 2 to 45 years. Seventy-three percent were male, 24 % were female and the remaining 3 % were hijra. Migration history was available for 47 VCT clients (Table 1) of which 33 said that they had worked abroad; six were presumed to have been related to partners who had worked abroad and eight were non-migrants. Most of the migrants had traveled to the Middle East (UAE, Saudi Arabia, Qatar). Some of them worked in South Asia (Pakistan and India) and South East and East Asia (Malaysia, Singapore, Thailand, Vietnam, South Korea), and the United States. All migrant workers claimed to have purchased sexual services from foreign women; several of them also had numerous risk factors, such as using injectable drugs, having intercourse with other men, and receiving blood transfusions while overseas. Migration history was not available for any of the 11 surveillance participants.

Among the non-C HIV-1 strains, subtype A, G and related recombinants were the most predominant (n = 50, 79.3 %) followed by subtype B and related recombinant (n = 7, 11.1 %), subtype D and related recombinant (n = 6, 9.5 %). A total of three dendrograms were constructed to determine their genetic relatedness among HIV-1 strains.

No	Specimen ID	Sex[1]	Age (years)	Group	Subtype (gagp17/p24)	Migration history	Sequence analysis			
							Most similar strain	country of origin	Accession	% nt[16] Identity
1	02BD054*	M	30	VCT[2]	A1	NA[8]	CY178	Cyprus	EU673446	96.2
2	02BD060	M	36	VCT	A1	UAE	BCCFE_HOMER_HIV_GAG_3145	Canada	EU242202	91
3	02BD061	M	38	VCT	A1	UAE	92UG_029	Uganda	AY713407	93
4	02BD062	F	35	VCT	A1	UAE(spouse)[9]	92UG_029	Uganda	AY713407	91
5	03BD008	M	35	VCT	A1	UAE	ML603-24.7.95	Kenya	EF164299	94
6	03BD026*	M	22	VCT	A1	Non-migrant	00KE_KER2009	Kenya	AF457053	94.6
7	05BD053	M	26	VCT	A1	UAE	HIV-1 clone 2814	Kenya	GQ432678	90
8	05BD059	F	20	VCT	A1	Non-migrant	HIV-1 clone 749	Kenya	GQ430613	89
9	04BD062*	F	19	VCT	A1	India	04_0566GT	Uganda	AY803394	93
10	04BD071*	F	20	VCT	A1	Non-migrant	CY178	Cyprus	EU673446	95.2
11	04BD130*	H	26	Hijra[3]	A1	NA	00KE_KER2009	Kenya	AF457053	95.2
12	04BD131*	H	25	Hijra	A1	NA	00KE_KER2009	Kenya	AF457053	95.2
13	02BD040*	M	40	MSM[4]	A1	NA	00KE_KER2009	Kenya	AF457053	94
14	99BG26	M	32	PWID[5]	B	NA	04CNLN130	China	EF122504	96
15	03BD067*	M	44	VCT	B	USA	8820638	Australia	AY857153	93.3
16	04BD004	M	31	VCT	B	Singapore	H61	Spain	DQ854715	93
17	04BD043*	M	34	VCT	B	Malaysia	CNHLJBF03009	China	EU131828	93.9
18	05BD051	M	26	VCT	B	Malaysia	2669	Russia	EF121245	94
19	04BD014*	M	32	VCT	D	S.Arabia	GT796	East Guinea	AY579680	91.5
20	03BD064	M	37	VCT	D	S.Arabia	03-5432NY	Uganda	AY803362	88
21	03BD068	M	35	VCT	D	S.Arabia	CA04	Cameroon	AF247517	93
22	05BD077	F	32	VCT	D	UAE (spouse)	ELI	D.R. Congo	K03454	93
23	02BD071*	M	40	VCT	G	S. Arabia	95LB11	Liberia	AF196694	95.5
24	02BD072*	F	28	VCT	G	S. Arabia (spouse)[10]	95LB11	Liberia	AF196694	98.5
25	02BD073*	M	30	VCT	G	S. Arabia	92NG003	Nigeria	U88825	94.5
26	03BD009	F	2	VCT	G	Non migrant	89SM_145	Somalia	AY713415	92
27	03BD022*	M	30	VCT	G	S. Arabia	95LB11	Liberia	AF196694	94.8
28	02BD063*	M	40	VCT	G	NA	92NG003	Nigeria	U88825	93.6
29	03BD058	M	38	VCT	G	S. Arabia	92NG003	Nigeria	U88825	94

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No	Specimen ID	Sex[1]	Age (years)	Group	Subtype (gagp17/p24)	Migration history	Sequence analysis			
							Most similar strain	country of origin	Accession	% nt[16] Identity
30	04BD060	M	40	VCT	G	S. Arabia	92NG003	Nigeria	U88825	91
31	05BD072	M	33	VCT	G	S. Arabia	92NG003	Nigeria	GU458665	93
32	02BD058*	F	26	VCT	CRF01_AE	NA	93TH054	Thailand	AB220945	97.9
33	02BD059	M	30	VCT	CRF01_AE	NA	00CMNYU1162	Cameroon	EF087995	93
34	02BD066*	M	23	VCT	CRF01_AE	Malaysia	pJPDR1741AE25	Japan	AB253640	94.8
35	02BD070*	M	38	VCT	CRF22_01_A1	S. Arabia	00CMNYU1162	Cameroon	EF087995	93.3
36	03BD075*	M	29	VCT	CRF01_AE	Malaysia	99TH.OUR044I	Thailand	AY358042	95.4
37	03BD021	M	28	VCT	CRF01_AE	S. Arabia	00CMNYU1162	Cameroon	EF087995	91
38	03BD027*	M	27	VCT	CRF22_01_A1	S. Arabia	00CMNYU1162	Cameroon	EF087995	93.3
39	04BD002*	M	31	VCT	CRF01_AE	Malaysia	06MMYKLD46	Malaysia	EF495062	98.2
40	04BD003*	F	20	VCT	CRF01_AE	Malaysia (spouse)[11]	06MMYKLD46	Malaysia	EF495062	98.5
41	04BD009	M	36	VCT	CRF01_AE	NA	C051M2P2350	Thailand	GU458736	93
42	04BD017	M	35	VCT	CRF01_AE	Malaysia	07LN184	China	FJ531435	94
43	04BD018*	M	25	VCT	CRF01_AE	Singapore	93TH054	Thailand	AB220945	96
44	04BD026	M	30	VCT	CRF01_AE	Multiple countries[12]	C051M1P579	Thailand	GU458520	95
45	05BD057	M	30	VCT	CRF01_AE	Singapore	C057M2P2430	Thailand	GU458743	94
46	04BD136*	M	30	PWID	CRF01_AE	NA	pCM235	USA	AF259955	97
47	00BD033	F	21	FSW[6]	CRF01_AE	NA	C116M1P2101	Thailand	GU458715	92
48	03BD112	F	24	FSW	CRF01_AE	NA	HCM309	Vietnam	AB044063	98
49	02BD047*	F	18	FSW	CRF01_AE	NA	144.1	Australia	EF116320	97.9
50	01BD020*	M	28	STI[7] patients	CRF02_AG	NA	J11243	S. Arabia	DQ375306	94.8
51	03BD074*	M	27	VCT	CRF02_AG	NA	p03GH189AG09	Ghana	AB286861	96.5
52	04BD005*	M	35	VCT	CRF02_AG	S. Arabia	LBV23.10	Gabon	L11779	92.1
53	04BD063*	M	45	VCT	CRF02_AG	S. Arabia	CM52885	Cameroon	AF377954	93.5
54	04BD070*	M	32	VCT	CRF02_AG	Multiple countries[13]	01IC-PCI127	Ivory Cost	AJ866558	93.5
55	03BD016	M	40	VCT	CRF09_cpx (CRF02, A, U)	Multiple countries[14]	95SN1795	Senegal	AY093603	89
56	03BD117	F	35	FSW	CRF09_cpx (CRF02, A, U)	NA	J11233	Saudi Arabia	EU697906	95

No	Specimen ID	Sex[1]	Age (years)	Group	Subtype (gagp17/p24)	Migration history	Sequence analysis			
							Most similar strain	country of origin	Accession	% nt[16] Identity
57	04BD031	F	20	VCT	CRF10_CD	S. Arabia (spouse)	96TZ-BF110	Tanzania	AF289550	89
58	04BD067*	M	42	VCT	CRF10_CD	Multiple countries[15]	GT796	Equatorial Guinea	AY579680	88.1
59	05BD074	M	32	VCT	CRF13_cpx (A, CRF01, G, J, U)	S. Arabia	96CM-1849	Cameroon	AF460972	94
60	04BD064	M	45	VCT	CRF14_BG	NA	X397	Spain	AF423756	92
61	03BD012	M	30	VCT	CRF15_01.B	Non-migrant	06MMYKLD46	Malaysia	EF495062	95
62	03BD017*	F	27	VCT	CRF15_01.B	Malaysia	06MMYKLD46	Malaysia	EF495062	97
63	00BD002*	M	28	MSM	CRF25_A,G,U	NA	J11451	S. Arabia	DQ375319	97

Table 1. HIV-1 non-C subtypes and their genetic and demographic information

**Sequence included in the tree; [1]M, male; F, female; H, hijra (transgendered people); [2]VCT, voluntary counselling and testing unit; [3]Hijra, transgendered people; [4]MSM, males who have sex with males; [5]PWID, People who inject drug; [6]FSW, female sex workers; [7]STI, sexually transmitted infections; [8]NA, history not available; [9]Spouse of a migrant who visited UAE; [10]Spouse of a migrant who visited Saudi Arabia; [11]spouse of a migrant who visited Malaysia; [12]South Korea, Malaysia, Singapore, Thailand, and Vietnam; [13]Saudi Arabia, UAE, India, and Pakistan; [14]Saudi Arabia and India; [15]Qatar and Saudi Arabia; [16]nt, nucleotide.*

HIV subtype A or G and related recombinant. The first dendrogram includes HIV-1 subtypes A1, G and their related recombinants AE, AG and AGU (Figure 1). The two identical A1 strains 04BD130 and 04BD131 from hijra were closely related to MSM strains 04BD026, 02BD040, and clustered in the same branch with a Kenyan strain. A high nucleotide identity (96 %) amongst the A1 strains from MSM and hijra suggests close sexual links between these groups. The other Bangladeshi A1 strains 04BD062, 02BD054, and 04BD071 clustered with strains from Africa.

Eight Bangladeshi CRF01_AE strains clustered with Asian lineage which includes also Bangladeshi female sex worker strains 02BD047 and BG33.1. Five of them returned from Malaysia and Singapore and they all said that they had bought sex from female sex workers while abroad. The remaining strains were identified from a female sex worker, a housewife, and a male PWID. They were also similar to Asian strains (Figure 1).

Two subtype G strains (02BD071 and 02BD072 with 98.5 % nucleotide identity) were identified in a married couple and clustered together with a Nigerian strain 01NGPL0567. The husband was a returnee migrant worker from Saudi Arabia who had bought sex from a female sex worker while working abroad. The other two subtype G strains, 02BD063 and 03BD022 placed in a different branch with an Italian strain IT031. The fifth strain 02BD073 which was identified from a male returnee migrant worker from Saudi Arabia, clustered in another separate branch distantly related to other Bangladeshi strains and was placed with a West African strain (M12259 from Congo). Bangladeshi CRF02_AG strains were closely related to each other and clustered with strains from Saudi Arabia, Equatorial Guinea, and the Ivory Coast. The other two CRF22_01_A1 strains (03BD027 and 02BD070) from two returnee male migrant workers from Saudi

Arabia clustered with African strains (01CM.0001BBY from Cameroon). Only one CRF25_AGU strain was identified which was very similar to a Saudi Arabian strain.

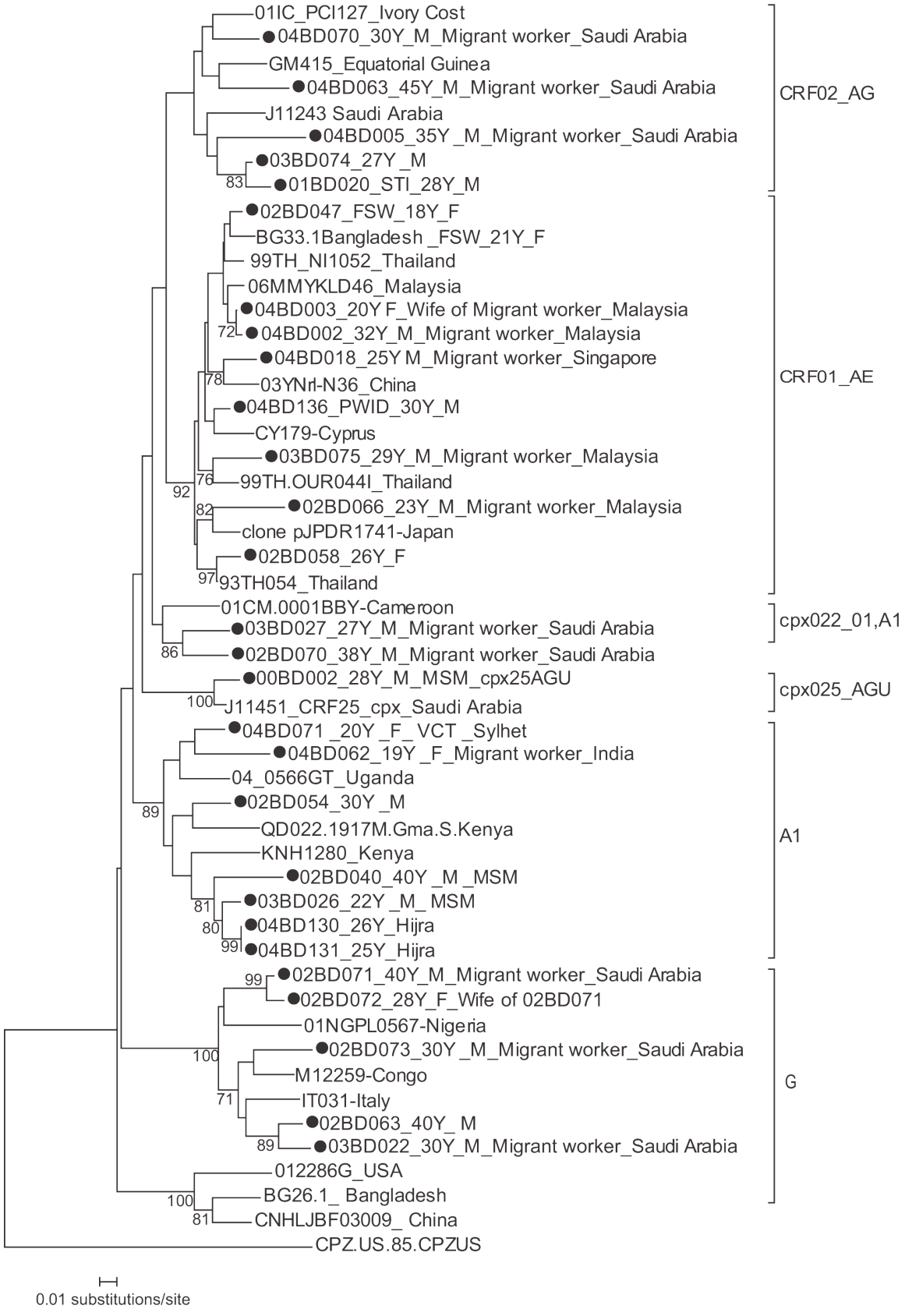

Figure 1. *Neighbor-joining phylogenetic tree based on nucleotide sequences of the partial gag encoding gene (349 nt bases) for HIV-1 subtype A1, G, CRF01_AE, CRF02_AG, CRF22_01_A1, and CRF25_AGU. The numbers adjacent to the nodes represent the value of bootstrap support (100 replicates) for*

the clusters to the right of the node. Bootstrap values lower than 70% were not shown. The dendrogram is rooted using simian immunodeficiency virus CPZ.US.85. M, male; F, female. The Bangladeshi strains are with a filled circle.

HIV subtype B and its related recombinant. Figure 2 shows that two Bangladeshi subtype B strains were placed in different branches; one (02BD067) with an American and another (04BD043) with Asian strains. The single strain of CRF15_AE/B (03BD017) from a 27-year-old female who lived in Malaysia with her husband clustered with a Malaysian strain. Her husband was also infected with the virus and had died from AIDS.

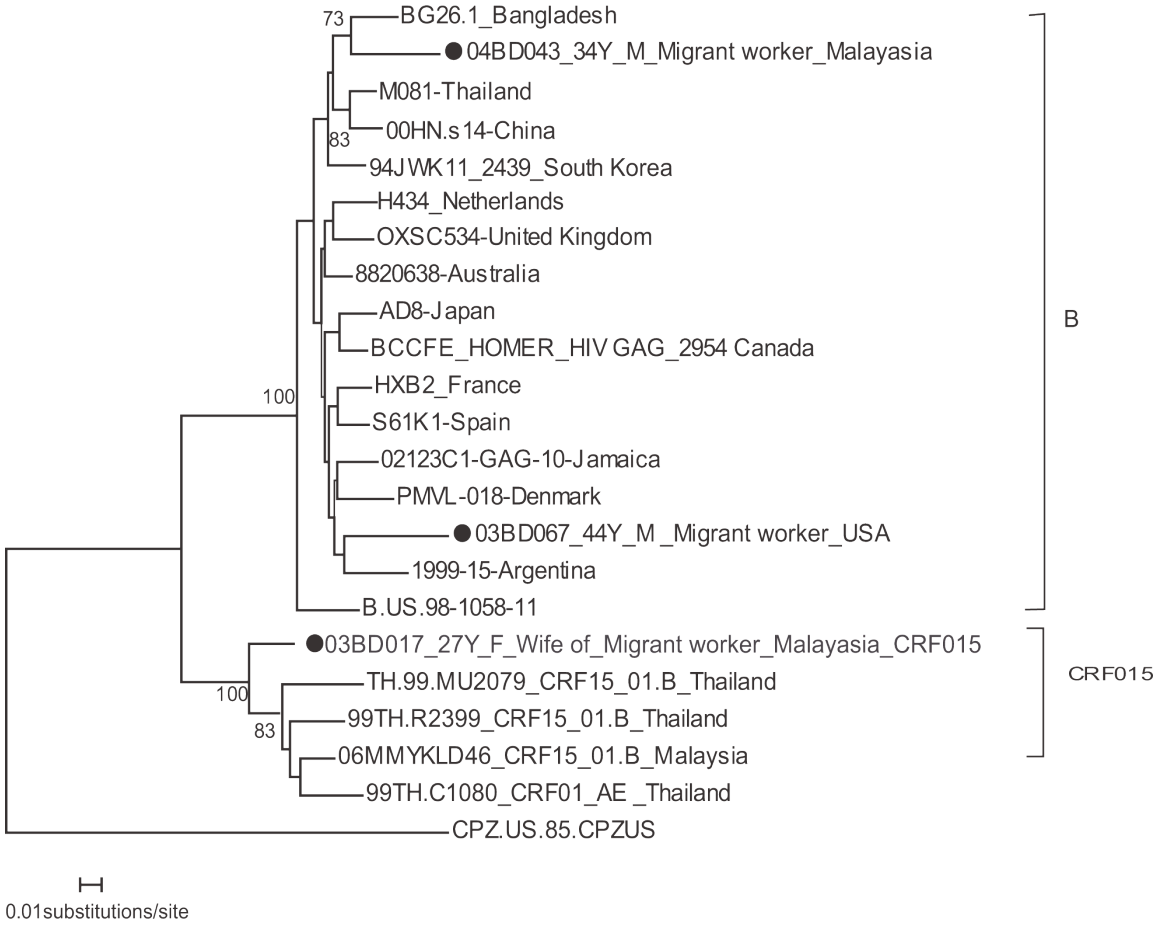

Figure 2. *Phylogenetic tree of HIV-1 subtype B and CRF15_AE/B based on partial gag gene. M, male; F, female. The Bangladeshi strains are with a filled circle.*

HIV subtype D and its related recombinant. Figure 3 shows that subtype D strain, 04BD014 was identified from a returnee migrant worker from Saudi Arabia who had a history of blood transfusion there and as expected clustered with two African strains (GT796 and 01CM_4412 HAL). The CRF10_CD strain 04BD067 clustered with Tanzanian strain 96TZ_BF061. This strain was identified in a male returnee migrant who worked in Qatar and Saudi Arabia for 17 years and had sex with female sex workers in Qatar.

We were able to sequence *env* genes from five sufficiently available samples and found that the *env* subtyping results perfectly matched with the *gag* subtyping assignment (Table 2).

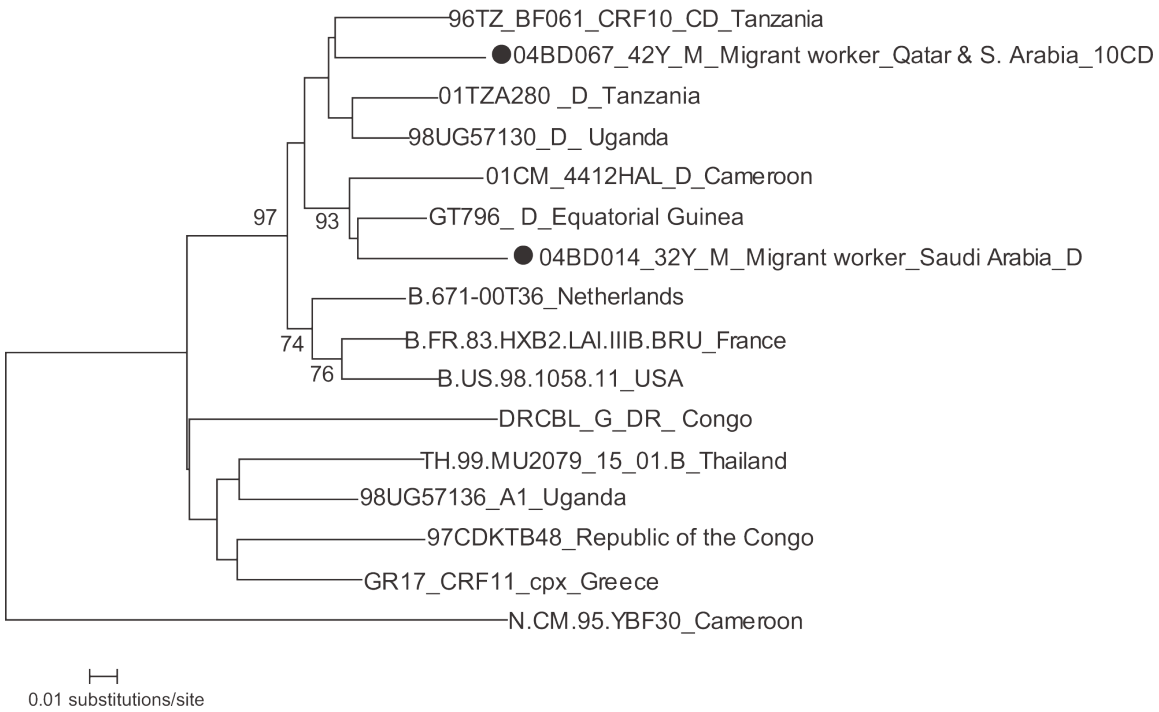

Figure 3. *Phylogenetic tree of HIV-1 subtype D and CRF10_CD). The dendrogram is rooted using HIV-1 group N (N.CM.95.YBF30). M, male; F, female. The Bangladeshi strains are with a filled circle.*

SL#	Specimen ID	Sex	Age(years)	Source	Suptype(*gag* p17/24	Suptype(*env* C2/V3/C4
1	02BD054	Male	30	VCT	A1	A1
2	02BD040	Male	40	MSM	A1	A1
3	02BD066	Male	23	VCT	CRF01_AE	CRF01_AE
4	03BD075	Male	29	VCT	CRF01_AE	CRF01_AE
5	02BD047	Female	18	FSW	CRF01_AE	CRF01_AE

Table 2. HIV-1 subtype determination by *gag* and *env* region

Discussion

This study investigated the non-C subtype of HIV-1 strains in Bangladeshi individuals and their transmission by using phylogenetic analysis along with demographic data. While considering the estimated low prevalence of HIV-1 in Bangladesh and hence the small sample size, the level of HIV-1 strain diversity was very high. The different patterns were illustrated by the phylogenetic analysis, which revealed that the HIV-1 strains shared many branches with other strains from throughout the world. Most of those whose strains were discovered had lived for work purposes in Middle Eastern, South Asian, or South East Asian countries. The main way that HIV spreads appears to be heterosexual, with different risks for a couple. Between Bangladesh and the Middle East, particularly Saudi Arabia, there is a sizable labor market. Our study provides evidence that many migrant workers sampled here were primarily infected with strains while buying sex from female sex workers in Saudi Arabia, however, these strains were genetically very close to African strains. This could be

explained by the fact that most of the Saudi Arabian strains originally were imported from African countries due to close geographical vicinity and commercial interactions between these countries[9]. At the same time, similar viruses were detected in the spouses of returnee migrant workers and in Bangladeshi female sex workers confirming that the migrant workers play a key role in spreading the viruses in the local community[10].

Genetic recombination among HIV-1 strains is a fundamental property that can be distinguished by analyzing the complete genome or multiple genes (*i.e. gag, pol,* and *env*). Analysis of only one gene may fail to accurately characterize the recombinant subtypes. Since our findings are based on only the *gag* gene sequence, we might have failed to characterize some recombinant strains accurately. However, further characterization using *env* or *pol* genes was not possible because this study used a small number of leftover samples collected through other studies.

Conclusions

With globalization and movements of people across borders, new and diverse strains of non-C HIV subtypes especially CRFs are regularly being introduced in Bangladesh, which have the potential to spread within the country through the networks of non-commercial and commercial sex. The ongoing targeted intervention programs in Bangladesh which are geared toward KPs[11][12] should include returnee migrant workers to minimize the spread of HIV in Bangladesh.

Methods

Sample collection. HIV non-C subtype strains were detected from leftover blood samples collected for the national HIV surveillance among KP in Bangladesh and the VCT Unit of icddr,b. These samples were collected and stored at -20°C as described in our previous paper[8]. All study participants provided informed, written agreement prior to blood collection, and in the instance of the one child who tested HIV positive, parental consent was also acquired. For those who couldn't read, the summary of the consent form was read, and for those who couldn't sign, a left thumbprint was taken.

Subtyping. Using nested polymerase chain reaction (PCR), proviral DNA was isolated from whole blood samples and amplified at the junction (p17/p24) of the gag gene as previously described[8]. The amplified products were nearly 400 bp (corresponding to positions 836-1249 in the HXB2 reference strain). We also randomly amplified and sequenced seven strains for the *env* region to check whether they were truly recombinant. For the *env* region, the primer for the first round PCR was ED5/ED12 (corresponding to positions 6517-7771 in the HXB2 reference strain), and for 2nd round it was 826/308 (corresponding to positions 6967-7382 in the HXB2 reference strain). The basic PCR conditions were as follows: initial activation for 2 minutes at 95 degrees Celsius, then 35 cycles of 94 degrees Celsius for 30 seconds, 62 degrees Celsius for 45 seconds, 1 minute at 72 degrees Celsius, and a final extension for 5 minutes in a final volume of 50 µl. The secondary PCR conditions were as follows: initial activation for 2 minutes at 95 degrees Celsius, 35 cycles of 94 degrees Celsius for 30 seconds, 58 degrees Celsius for 45 seconds, 1 min at 72 degrees Celsius, and a final extension for 5 minutes in a final volume of 25 µl. The second cycle of PCR was conducted using the same reaction mixture and three microliters of the initial amplified product. Electrophoresis on a 1.5% agarose gel was used to identify the PCR products, and ethidium bromide staining was used to make them visible.

On an ABI 377 automated DNA sequencer, the amplified DNA was sequenced. Using Chromas 2.23 (Technelysium, Queensland, Australia), the chromatogram sequencing files were examined, and SeqMan II was used to create the consensus sequences (DNASTAR, Madison, WI). ClustalX1.81 was used to calculate multiple sequence alignments[13]. HIV genotyping tools (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) were used to subtype partial sequences of the *gag* and *env* genes (at least 300 nucleotide bases). A manual phylogeny using reference strains from the Los Alamos HIV sequencing library was used to confirm all subtypes (www.hiv.lanl.gov). Finally, 349 base pair sequences of 33 *gag* genes were evaluated. Sequences at the 5´ and 3´ ends

were excluded from the alignment for those that could not be aligned clearly due to the length variability of the sequences. The MEGA version 6 software program was used to perform phylogenetic analyses using the neighbor-joining approach and to determine genetic distances using the nucleotide p-distance model[14].

The accession numbers JX310758 through JX310790 (for the *gag* sequence) and KF421247-KF421251 were used to submit the nucleotide sequences to GenBank, NCBI (for *env* sequence).

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Conflicts of interest

There are no competing interests of the authors. The study's design, data collection and analysis, publication decision, and manuscript preparation were all done independently from the funders.

Ethics approval and consent to participate

Each study participant provided informed, written consent prior to blood collection; in the case of minors, consent was given by the parent or legal guardian. The summary of the consent form was read to those who couldn't read, and left thumbprints were taken from those who couldn't sign. This study has received permission from the icddr, Ethical B's Review Committee.

Authors' contributions

The experiments were conceptualized and designed by MSS; they were carried out in the lab by MSS; the data were analyzed by MSS; and the manuscript was written and revised by MSS. The final manuscript was read and approved by the author.

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Declarations

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