

## Regional spread of HIV-1 M subtype B in middle-aged patients by random *env*-C2V4 region sequencing

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**Abstract** A transmission cluster of HIV-1 M:B was identified in 11 patients with a median age of 52 (range 26–65) in North-East Germany by C2V4 region sequencing of the *env* gene of HIV-1, who—except of one—were not aware of any risky behaviour. The 10 male and 1 female patients deteriorated immunologically, according to their information made available, within 4 years after a putative HIV acquisition. Nucleic acid sequence analysis showed a R5 virus in all patients and in 7 of 11 a crown motif of the V3 loop, GPGSALFTT, which is found rarely. Analysis of formation of this cluster showed that there is still a huge discrepancy between awareness and behaviour regarding HIV transmission in middle-aged patients, and that a local outbreak can be detected by nucleic acid analysis of the hypervariable *env* region.

**Keywords** HIV transmission · Epidemiology · Cluster · Risky behaviour · Local outbreak · Middle-aged people

### Introduction

The route of sexual spread of human immunodeficiency virus (HIV) is known since 1981 [1]. Awareness of spread of this infectious agent in different parts and different people of Germany is variable, with a trend that especially young homosexual men (MSM) are predominantly affected [2].

The aim of this study was to find the route of transmission of HIV infection in a few patients who presented with AIDS or AIDS-like symptoms in a group of middle-aged people non-exposed to obvious epidemiological or individual risk factors.

### Patients

All patients, 10 males and 1 female, were born and/or living in a geographically restricted area around Greifswald. Patients' data are summarized in Table 1. Patients had been identified and analysed as HIV infected either according to their clinical AIDS-defining symptoms or after getting information that a sexual partner was found to be HIV infected. One of the patients was identified when he desired to donate blood.

Most patients could not explain how, when and where they acquired their HIV infection. Only one of the male patients named his HIV-infected source partner; he stated to be HIV antibody negative before his homosexual relationship started with the index patient. This index patient

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**Table 1** Demographic data, laboratory test results, and clinical parameters obtained from the patients included in this study

Patient number	Sex	Age at diagnosis	Viral load (cop/ml) at the first hospital visit	CD4 cells/ $\mu$ l at diagnosis	Coreceptor tropism R5/X4	V3 loop crown motif	Disease stage	Main symptoms
HGW1	M	61	48,000	550	R5	GPGRALYST	C1	Atrophic dermatitis
HGW2	M	48	310,000	270	R5	GPGSALFTT	C3	Weight loss Neurologic
HGW3	M	46	125,000	150	R5	GPGRALFTT	C3	Neurologic
HGW4	M	57	72,000	120	R5	RPGSALFTT <sup>a</sup> GPGSALFTT	C3	Marasmus Zoster
HGW5	F	55	41,000	250	R5	GPGSALFTT	A2	None
HGW6	M	56	87,000	330	R5	GPGSALFTT	C3	Weight loss Pemphigus
HGW7	M	26	330,000	170	R5	GPGRALYST	C3	Weight loss
HGW8	M	39	29,000	600	R5	GPGRALLTT	C2	Candidiasis
HGW9	M	65	11,000	650	R5	GPGSALFTT	A1	None
HGW10	M	64	32,000	540	R5	GPGSALFTT	C2	None
HGW11	M	52	280,000	45	R5	GPGSALFTT	C3	Weight loss Candidiasis

<sup>a</sup> Both sequences were found in this patient, circulating in approximately equal amounts

was neither available for interview nor his blood for nucleic acid sequence analysis. Additionally, a person who might be the real source of spread of this HIV infection could not be traced, despite several efforts made.

## Materials and methods

For nucleic acid sequence analysis, EDTA blood was drawn by venepuncture. HIV antibody was tested from the EDTA-plasma with a commercially available kit (Enzygnost, Dade-Behring, Marburg, Germany). Presence of antibody was confirmed by immunoblot (HIV-Innolia, Innogenetics, Ghent, Belgium). Viral load was determined by the Amplicor Monitor test (Roche, Alameda, CA, USA; no longer available). CD4 cells were measured by FACS analysis (Becton–Dickinson, Heidelberg, Germany).

RNA was extracted from EDTA-plasma according to the recommendation of the manufacturer (Qiagen, Hilden, Germany). RNA was transcribed to DNA by reverse transcription using a one step RT-PCR (Superscript, Invitrogen, Carlsbad, CA) at 50°C for 30 min, followed by a nested PCR using Taq polymerase (Platinum Taq polymerase, Invitrogen, Carlsbad, CA). PCR-cycling conditions were denaturation at 92°C for 30 s, annealing at 50°C for 90 s, and elongation at 72°C for 90 s, cycling 30 times. Final elongation was done at 72°C for 7 min.

Primers used were (in 5' to 3' direction)

5env 6901: CCT CAg CCA TTA CAC Agg CCT gTC CAA Ag (HXB2 6818-6846) and 3env 7681: ATA TAA

TTC ACT TCT CCA ATT gTC (HXB2 7792-7616) for the first amplification step; and 5env 7001: gCA CAg TAC AAT gTA CAC ATg gAA (HXB2 6949-6974) and 3env7401: TTA CAg TAg AAA AAT TCT CCT C (HXB2 7380-7361) for the second amplification step.

Amplificates of the second/nested PCR were analysed by 2.5% agarose gel electrophoresis in the presence of ethidium bromide, and the specific band was after purification subjected to the dideoxynucleotide sequencing cycles [3] using the AbiPrism labelling kit and the 301 sequence analyser (Applied Biosystems, Weiterstadt, Germany). Sequences were analysed and aligned with the Sequence Navigator or BioEdit (<http://www.mbio.ncsu.edu/BioEdit>) program. Construction of the phylogenetic tree was performed using 372 nucleotides of the C2V4 region including the V3 loop by the Treecon and Mega 4 program [4, 5]. Pathogenicity index and X4/R5-coreceptor use was calculated according to the formula of Briggs et al. [6] weighing the charge of acidic and basic amino acid residues in the V3 loop.

Mutation rate was calculated using an average rate of 0.5% per year in the V3 loop published 1990 by Balfe et al. [7] for recently infected haemophiliacs, which is close to the data given by Ji and Loeb [8] of 2 in 10<sup>4</sup> errors per base per replication cycle in the *VI gene* and of Salazar-Gonzales et al. [9] of 1.7 × 10<sup>-5</sup> substitution per site per day in the whole *env gene* in asymptomatic individuals, which is 0.6% per year. The average transition/transversion rate within the *env/V3* was given with 1.42 [10], which is higher than in other parts of the *env gene*.

## Results

### Patients

In comparison to other epidemiological studies that reported a clustering of HIV transmission, the high age of the majority of the patients, in average 52, range 26–65, is remarkable; additionally, it is striking that only one female was involved, who was most probably infected by her husband. Nine of the 11 patients showed a clinical presentation compatible with advanced signs of immunodeficiency.

### Analysis of the nucleic acid sequence of the C2V4 region

The crown motif of the viruses could be ranked in two groups, GPGSALFTT, which was found in seven patients—in patient HGW4 an nearly equal mixture of the N terminal G and R was found—and GPGRALYST/GPGRALYTT in the remaining four patients (Table 1). The codon AGT for the amino acid S (serine) was conserved in all sequences while a codon AGG (twice) and AGA (once) was used for R (arginine) in the GPGS or GPGR crown motif, indicating that an exchange of G or A by T in the third position was the drive for selection of serine. The codon of the aspartic acid (D) in amino acid position 321, within the C-terminal part of the V3 loop, was deleted in all sequenced strains of these patients (see below for three sequences and in Table 2), which is a rare event. However, it was found initially in the LAI/BRU virus (clone HXB2), as shown below (the one letter code of amino acids was used).

amino acid position/ strain	296	331
HXB2:	CTRPNNNTRKRIRIGPGRAVFTIG-KIGNMRQAH	C
WEAU:	CTRPNNNTRKKITLGPGRVLYTTGDIIGDIRRAHC	C
HGW4	CTRPNNNTRRGVHVRPGSALFTTH-IIGDIRQAH	C
HGW5	CTRPNNNTRRGVHVGPGSALFTTH-IIGDIRQAH	C
HGW7	CTRPNNNTRKGVHVGPGRALYTTTH-IIGDIRQAH	C

### Mutation rate and duration of HIV infection

Analysis of the nucleic acid sequences of the couple HGW4 and HGW5 revealed 80 mutations per 340 nucleotides in the C2V4 region. Considering a mutation rate in 2 individuals and an increase of mutations in the HGW4 patient with severe symptoms of AIDS of 1% per year [11], the time point of infection could have been 12 years ahead—around 1994. In contrast, the information given by

the male patient on his putative infection time was 2002. Another, homosexually active, patient of this cluster focused his time point of infection around 7 years ago, which is more close to the 12 years time point of divergence of the cluster of these patients.

### Phylogenetic tree

Compared to the more than 100 HIV-1 subtype B strains analysed in our laboratories, all C2V4 sequences of the 11 patients clustered together and were thus epidemiologically linked (Fig. 1). Boot strap analysis revealed a value 57–86 of 100 for the branching of this cluster from all other subtype B sequences, running 1,000 comparisons. There was no difference between the tree constructed by the Treecon program or the Mega4 program, in both trees the cluster of the 11 sequences segregated separately from all other HIV-1 M B-subtypes. Within the 177 nucleic acid sequences of the C2V4 region analysed, and within all sequences available at the Los Alamos Data Base (update 2007), there was no other nucleic acid sequence identical to that of the majority of this cluster.

## Discussion

This study describes a further cluster of HIV transmission to 11 patients in a locally restricted area identified by nucleic acid sequencing of part of the *env* gene region. By the same method, an intrafamilial cluster of HIV-1 transmission involving three persons had been identified in France in 1998 [12], as well as heterosexual transmission of HIV-1 in Senegal [13] and in Belgium by a man originating from Rwanda who infected from 1992 to 1996 six recipients [14]. By nucleic acid sequence analysis of the *pol* gene, not the *env* gene, the transmission of multiple drug-resistant HIV in a cluster was determined [15]. Earlier reports describe the transmission of HIV-1 to 11 middle-aged women by one man in Belgium 1989 [16], from one man to 13 women in 2002 in New York [17]. Genotypic signature characteristics with a lower charge of the V3 loop in the recipients had been described in a study in Uganda [18], this charge difference was not found in the cluster described here. Regional HIV outbreaks have been reported more recently in Quebec [19] especially during the acute/early period of HIV infection identifying at least 7 (range 2–17) transmission clusters involving 293 people with a medium age between 34 and 39 ± 10. Another study involved approximately 65 persons of whom at least three were infected heterosexually during a short period in Los Angeles in 2004 in the adult film industry where the index patient had an age of 40 [20].

**Table 2** C2V4 amino acid sequences of the patients involved in the cluster, aligned to the HXB2 sequence

HGW1: QLLNGLSLAEEEVVRSENFTDNAKTIIVQLTESVKINCRP--NNNT-RKGVHV--GPGRALYS--TH-II--GDIRQAHNCISKANWNTLKKIASKLRE--HFENKPIVLIQSS--GGDPKIV

HGW2: QLLNGLSLAEEEVVRSENFTDNAKTIIVQLNESVINCTSP--NNNT-RRGIHT--GPGSALFT--TH-II--GDIRQAYCNISRAKWNATLQKVATKLGE--QYSSTKIIFNSSS--GGDVEIV

HGW3: QLLNGLSLAEEEVVRSENFTDNAKTIIVQLNESVEINCARP--NNNT-RNSVHV--GPGRALFT--TH-II--GNIRQAHNCISRTNNTAALKNVATKLRE--QFNSTIIVFNSSS--GGDLLV

HGW4: QLLNGLSLAEEEVMIRSENFTDNVKTIIIVQLNESVQINCRP--NNNT-RRGVHV--GPGSALFT--TH-II--GDIRQAHNCINRTKWNDAKKIATKLIE--QFNSTIIVFNSSS--GGDLEIE

HGW5: QLLNGLSLAEKEVMIRSENFTDNVKTIIIVQLNESVEINCRP--NNNT-RRGVHV--GPGSALFT--TH-II--GDIRQAHNCISRAKWNATLQKVIATKLRE--QFNSTIIVFNSSS--GGDIEIV

HGW6: QLLNGLSLAEEEVMIRSKNFTNNVDTIIIVQLNESVEINCRP--NNNT-RQSVHV--GPGSALFT--TN-II--GDIRQAHNCISRAKWNATLQKIATKLGE--RYNRTIXXFTSSS--GGDIEIT

HGW7: QLLNGLSLAEEEVVRSENFTDNAKTIIVQLNETVKINCRP--NNNT-RKGVHV--GPGRALYT--TH-II--GDIRQAHNCISRAKWNATLQKVATKLRE--QFNSTIIVFNSSS--GGDLEIV

HGW8: QLLNGLSLAEEEVVRSENFTDNVKTIIIVQLNESVTINCRP--NNNT-RKGVHV--GPGRALLT--TH-II--GDIRQAYCNISRAEWSAALKKXVTKLRE--QFNSTIIVFNSSS--GGDLLIV

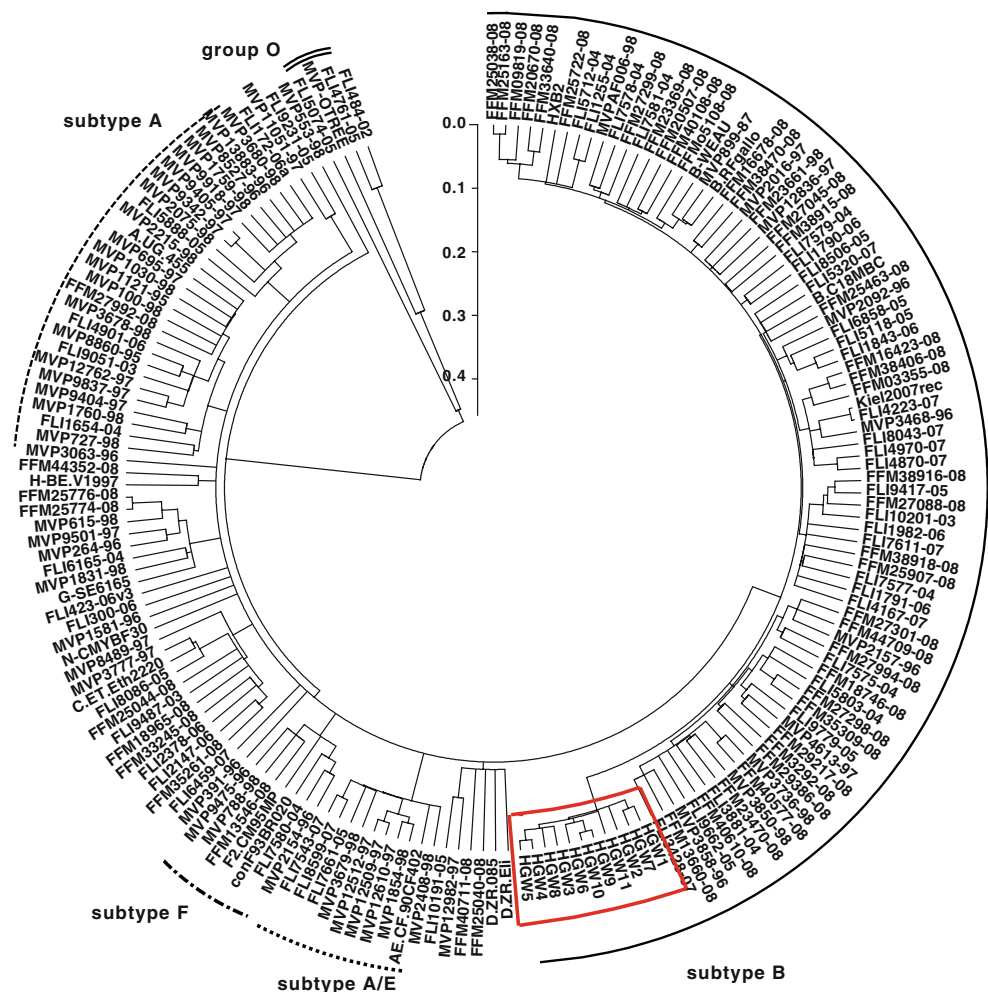
HGW9: QLLNGLSLAEGEVMIRSKNFTNNADTIIIVQLNESVAINCRP--NNNT-RKGVHV--GPGSALFT--TH-II--GDIRQAHNCISRTQWNTALQKIVTKLRE--RYNGXITIFNSSS--GGDLEIT

HGW10: QLLNGLSLAEGEVMIRSKNFTNNADTIIIVQLNESVTINCRP--NNNT-RKGVHV--GPGSALFT--TH-IM--GDIRQAHNCISRTQWKATLQKIVTKLRE--QYNGXIIIFNSSS--GGDLVIT

HGW11: QLLNGLSLAEEEVVRSEXTDNVKTIIIVQLNESVEINCRP--NNNT-RRSVHV--GPGSALFT--TN-II--GDIRQAHNCISRAKWNATLQKVAVKLKE--QFNSTIIVFNSSS--GGDPEIV

The one letter code of amino acid sequences was used. In the middle, the GPGR motif of the crown of the V3 loop may be seen. The sequences start at the amino acid position 258 of HXB2 according to the sequence counting of Los Alamos data base (21)

**Fig. 1** Phylogenetic tree of the nucleic acid sequences of 177 HIV's, including some reference sequences for comparative analysis of the HIV-1 group B sequences of the patients of the described cluster, drawn with the Mega 4 program. As may be seen in the lower part of the tree, marked with the red square, the cluster is formed by the 11 sequences (HGW1–11) and separated from all other sequences analysed in Greifswald (FLI), München (MVP) and Frankfurt (FFM). Roughly the position of subtype B strains (*bow on the right*), and of subtypes A, A/E and F (*dashed lines*) are given; the position of the 3 group O strains is indicated with a *double line* on the *left upper end* of the tree



According to the HIV sequence data generated, the 11 patients form a unique cluster, with V3 loop sequences that had not been described previously (see Fig. 1); since the V3 loop is hypervariable the C2V4 region was selected to

get valuable information. For the cluster analysis the choice of the HIV gene region is more important than the program used [21]. Further statistical methods, as maximum-likelihood or Bayesian approach, cannot support

relationship of various HIV when there is a strong virologic link [22]—as described here.

The crown motif of the V3 loop with the amino acid sequence GPGSALFTT, which was found in 7 of the 11 patients, has not been deposited in the Los Alamos Data Bank (<http://www.hiv.lanl.gov/>) [23]. Beneath 1,766 sequences available there were 4 similar subtype B sequences with the crown motif GPGSALxxx or xxGSALFxx one from Spain (B.ES.xLPM.MHO2.EF531331), one from Ecuador (B.EC.89.EC102.AY173960), and two from United States, indicating how rare this sequence motif is.

The variant amino acids of those cited strains are as follows:

HGW4	GPGSALFTT
B.ES.xLPM.MHO2.EF531331	-----Y-R
B.EC.89.EC102.AY173960	VG-----YA
B.US.LC04A18_FLQ1018.M90933	----S----
B.US.LC04A2_FLQ102.M90931	-----Y--

Analysis of the therapy naïve patients of this cluster yielded the following peculiarities: a median age of approximately 52, which is high compared to other studies [18, 20]. According to the information given by the patients, unawareness of any risky behaviour in the past or inability to realize where and when risky behaviour had happened, as has been reported in previous studies as well [16, 24]. All patients were carrying a R5 virus, despite the manifestation of full blown AIDS in 5 patients (see Table 1), and all viruses of this cluster had no mutations in the *pol*-gene linked to drug resistance of the protease and reverse transcriptase (data not shown).

Regarding this cluster, it is striking that only one female was involved, who was most probably infected by her husband. Additionally, 8 of the 11 patients were married. Despite the various efforts made the source—one or several index patients [19, 25, 26]—and the route of HIV-1 transmission—homosexual, bisexual, heterosexual—in this cluster is still unclear. To get access to the source of infection, a further described way for cluster analysis is by partner notification, without nucleic acid sequencing [25, 27, 28], which was not possible for these 11 patients investigated.

The HIV infection of these patients opens a considerable gap of knowledge concerning HIV transmission in a non-promiscuitive setting, since only one of the patients was willing or able to give information on his route of infection. Most of the patients involved were identified by deterioration of the immune system between 2005 and 2008. Low

awareness of the risk of HIV transmission is a fundamental cofactor in the spread of HIV within communities. There had been several, annually repeated efforts to rise the level of awareness focused on the social status, on ethnic minorities, on sexual preference, on education, and on prevention modes [16, 23, 24, 28, 29]. This low awareness is not country dependent, mostly independent of funding, but highly dependent on human behaviour [26, 30]. The cluster described in this paper shows that besides young people and predominantly young MSM in the western world also elderly people are at risk of HIV acquisition and unaware of their risky behaviour even after 25 years of HIV counselling and information [31] and that information campaigns should address all age groups.

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