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Impact of triplicate testing on HIV genotypic tropism prediction in routine clinical practice

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Abstract

Guidelines state that the CCR5-inhibitor Maraviroc should be prescribed to patients infected with R5-tropic HIV-1 only. Therefore, viral tropism needs to be assessed phenotypically or genotypically. Preliminary clinical trial data suggest that genotypic analysis in triplicate is associated with improved prediction of virological response by increasing the detection of X4-tropic variants. Our objective was to evaluate the impact of triplicate genotypic analysis on prediction of co-receptor usage in routine clinical practice. Samples from therapy-naive and therapy-experienced patients were collected for routine tropism testing at three European clinical centres. Viral RNA was isolated from plasma and proviral DNA from peripheral blood mononuclear cells. Gp120-V3 was amplified in a triplicate nested RT-PCR procedure and sequenced. Co-receptor usage was predicted using the Geno2Pheno$_{[coreceptor]}$ algorithm and analysed with a false-positive rate (FPR) of 5.75%, 10%, or an FPR of 20% and according to the current European guidelines on the clinical management of HIV-1 tropism testing. A total of 266 sequences were obtained from 101 patient samples. Discordance in tropism prediction for the triplicates was observed in ten samples using an FPR of 10%. Triplicate testing resulted in a 16.7% increase in X4-predicted samples and to reclassification from R5 to X4 tropism for four cases rendering these patients ineligible for Maraviroc treatment. In conclusion, triplicate genotypic tropism testing increases X4 tropism detection in individual cases, which may prove to be pivotal when CCR5-inhibitor therapy is applied.

Keywords: CCR5, CXCR4, genotypic, HIV-1, maraviroc, R5-tropic, tropism, X4-tropic
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Introduction

Maraviroc (MVC) is the first available antiretroviral drug targeting a human receptor. It binds to the CCR5 co-receptor thereby inhibiting replication of CCR5 using (R5-tropic) HIV-1 [1,2]. MVC has been approved for HIV-1-infected patients that exclusively harbour R5-tropic viruses and is licensed in Europe for therapy-experienced patients and in the USA for both therapy-experienced and therapy-naive patients. As MVC has no antiretroviral effect on strains using the CXCR4 co-receptor (X4-tropic), determination of co-receptor usage (viral tropism testing) is needed to exclude the presence of X4-tropic HIV-1 strains. For determination of viral tropism several phenotypic and genotypic assays have been developed. Among phenotypic tropism tests, the 'enhanced sensitivity Trofile™ assay' (ESTA; Monogram Biosciences, San Francisco, CA) is most often used [3,4]. However, for clinical centres, ESTA has several limitations: testing is only performed in California (USA), resulting in logistical problems, long turnaround time and high costs. Furthermore, the assay

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is only available in Europe for samples with HIV RNA ≥1000 copies/mL. For these reasons tropism testing is increasingly performed using genotypic assays.

Genotypic tropism tests analyse the sequence of the HIV-1 envelope gp120 variable 3 (V3) loop, the main determinant for co-receptor usage. To predict viral tropism the generated V3 sequences are interpreted using publicly available algorithms, such as $\text{Geno2Pheno}_{[\text{coreceptor}]}$ (G2P) and position-specific scoring matrices ($\text{PSSM}_{\text{X4-R5}}$) [5,6]. Genotypic tropism testing can be applied on population sequences obtained from either HIV RNA or HIV proviral DNA. The latter is recommended if HIV RNA levels are below the level of reliable amplification [7]. Population sequencing, the most frequently used method of genotypic tropism testing, is hampered by limited sensitivity for detecting minority X4-tropic strains in the quasi-species. As such, minority X4-tropic variants may remain undetected when they represent <10–25% of the total population [8–10].

Despite limitations in sensitivity compared with ESTA, population genotypic tropism testing demonstrated equal predictive value for virological outcome of MVC-containing therapy in antiretroviral naive individuals [11]. In this particular retrospective analysis a genotypic testing procedure was performed in triplicate to increase detection of minority X4 populations.

The rationale for performing genotypic tropism testing in triplicate, instead of using a single procedure as usually performed for resistance testing on *pol*, is based on differences in selective pressure on the viral envelope protein compared with *pol*, which are reflected by the nine-fold higher nucleotide substitutions/site/year in *env* [12]. The relatively high levels of variation in *env* may be better captured in a triplicate procedure.

In therapy-experienced patients, re-analysis of three clinical trials demonstrated that triplicate genotypic tropism testing increased the number of X4-predicted samples [13]. Preliminary data suggest that testing in triplicate has a beneficial effect on predicting clinical outcome of MVC-containing regimens [13].

However, in clinical cohort studies triplicate genotypic tropism testing is not performed routinely. Still a good correlation between genotypic tropism testing and ESTA in predicting virological outcome to MCV-containing therapy has been observed [14–17]. As such, the added value of triplicate testing in routine care is still under debate.

In the absence of a direct comparison of single and triplicate test procedures in clinical practice, the recently formulated European guidelines advise triplicate testing with a false-positive rate (FPR) of 10%. If single testing is performed then a more conservative FPR of 20% for RNA samples with a viral load <1000 copies/mL and for proviral DNA samples is recommended [7].

We investigated the influence of triplicate testing on tropism prediction during routine clinical practice in three European clinical centres.

Materials and Methods

Patient samples on which routine tropism testing was performed in clinical practice were randomly selected from three European centres. HIV-1 plasma RNA levels and counts of $CD4^+$ cells/mm^3 at nadir and at time of sampling were collected, HIV proviral DNA was not measured. HIV-1 pol subtyping was based on IDNS (Smartgene, Lausanne, Switzerland) or the Rega HIV-1 subtyping tool [18].

Viral RNA, DNA isolation

Viral RNA was isolated from 200–500 μL EDTA-plasma with the Viroseq HIV-1 sample preparation module (Abbott, Hoofdorp, the Netherlands) or a high pure viral RNA kit (Roche, Vilvoorde, Belgium). If no plasma was available or the HIV RNA level was below the level of amplification, proviral DNA was extracted from $1.0E^7$ peripheral blood mononuclear cells with QIAamp DNA Blood Mini Kit (QIAGEN, Madrid, Spain). For each sample, one isolation was performed. Subsequent processing of the samples, amplification and sequencing, were performed in triplicate. In each isolation and amplification round two or three negative controls were included, depending on the number of isolations and amplifications.

Viral RNA amplification

For amplification of the V3-loop, two in-house protocols were used. Protocol one; 10 μL of RNA, with primers 6206V3F 5′-AGAGCAGAAGACAGTGGCAATGAGAGTGA-3′, 7785R 5′-AGTGCTTCCTGCTGCTCCYAAGAACCC-′3 (Titan One Tube RT-PCR kit, Roche, Woerden, the Netherlands) for RT-PCR. Subsequently a nested-PCR was performed using primers 6658F 5′-TGGGATCAAAGCCTAAAGCCATGTG-′3, 7371R 5′-GAAAATTCCCCTCCACAATT-′3 (Expand High-Fidelity PCR-System, Roche, Woerden, the Netherlands). Sequencing was performed with primers 6957F 5′-GTACAATGTACACATGGAAT-′3 and 7371R or V3-4 5′-ACAGTACAATGTACACATGGAATTA-3′ and V3-3 5′-AATTCCCCTCCACAATTAAAASTGTG-3′ (Big dye Terminator Cycle seq kit v3,1, Applied Biosystems, Nieuwekerk ad IJsel, the Netherlands). Protocol two; for the RT-PCR 10 μL RNA and a mixture of the primers sense ENV_11 5′-GGATATAATCAGYYTATGGGA-3′, antisense ENV_22 5′-GGTGGGTGCTAYTCCYAITG-3′, sense-ENV1 5′-GAG-

GATATAATCAGTTTATGG-3′ and antisense-7294 5′-GGTGGGTGCTATTCCTAATGG-3′ (Titan One Tube RT-PCR kit, Roche, Vilvoorde, Belgium) were used. These primer mixtures cover a broader range of HIV variants The nested-PCR was performed using primers sense-ENV_33 5′-GAT CAAAGCCTAAARCCATGT-3′, antisense-ENV_44 5′-CTC CAATTGTCCYTCATHTYTCC-3′, sense-ENV2 5′-GA TCAAAGCCTAAAGCCATG-3′ and antisense-7238 5′-ACT TCTCCAATTGTCCCTCATAT-3′ with AmpliTaq DNA polymerase (Applied Biosystems, Halle, Belgium). Amplified product was sequenced with primers sense-6951 5′-AG YRCAGTACAATGYACACATGG-3′, sense-6690 5′-TCAA CHCAAYTRCTGTTAAATGG-3′ and antisense-7336 5′-ATT TCTRGRTCYCCICCYG-3′ (Big dye Terminator Cycle seq kit v3,1, Applied Biosystems).

Proviral DNA amplification

For amplification 3 μL DNA was used to amplify full-length envelope with Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen, Barcelona, Spain) using primers 5677U24 5′-ATG GCTTAGGGCAACATATCTATG-3′ and 9687L24 5′-CTGAGGGATCTCTAGTTACCAGAG-3′ or primers 5954U29 5′-CACCTAGGCATCTCCTATGGCAGGAA GAAG-3′ and 8904L22 5′-GTCTCGAGATACTGCTCCC ACCC-3′. Nested-PCR using primers 5954U29 5′-CACCTA GGCATCTCCTATGGCAGGAAGAAG-3′ and 8904L22 5′-GTCTCGAGATACTGCTCCCACCC-3′ or 6373U22 5′-CCACTCATTTTGTGCATCAGA-3′ and 7855L25 5′-AAY TGTCTGCCTGTACCGTCAGCG-3′ (Platinum® *Taq* DNA Polymerase High Fidelity, Invitrogen). Sequenced using primers 7002U20 5′-CTGTTAAATGGCAGTCTAGC-3′ and 7374L25 5′-AGAAAAATTCYCCTCYACAATTAAA-3′, or 6959U25 5′-ACAATGYACACATGGAATTARGCCA-3′ and 7365L21 5′-CCCCTCCACAATTAAAACTGT-3′ (Big dye Terminator Cycle seq kit v3.1, Applied Biosystems, Madrid, Spain).

Tropism prediction

Nucleotide sequence tropism prediction was performed *in silico* using G2P and an FPR of 5.75%, 10%, 20% and according to the current European guidelines [7]. For web-PSSM$_{X4\text{-}R5}$ the amino acid sequence was used. In the case of mixtures all possible amino acid sequences were analysed and the highest value was reported (R5 prediction: $\leq$–6.69, X4: $\geq$–2.88, the 11/25 rule was applied at intermediate values) [19]. If an isolate was predicted to be X4-tropic in at least one of the three tests the viral population was reported to be X4-tropic.

Statistical analysis

Pearson chi-squared test was used to compare the ratio X4-predicted sequences between low (<350) and high ($\geq$350) CD4$^+$ cells/mm^3, to compare the ratio of X4-predicted sequences of G2P versus PSSM$_{X4\text{-}R5}$, and to evaluate the ratio and number of X4-predicted sequences, and samples between single, duplicate or triplicate testing. Mann–Witney *U*-test was used to compare viral RNA load between samples with or without amplification failure. Furthermore, data were randomized with randperm in MATLAB 2010b. Values below 0.05 were regarded statistically significant.

Results

The majority of 101 patients (70) were infected with subtype B, followed by: C (6), CRF02_AG (6), CRF01_AE (5), A (4), A1 (2), G (1), H (1), J (1), CRF15_01B (1), CRFAB (1), CRF30 (1), CRF18_cpx (1) and one unclassified strain. The median viral load was 8.35 E3 copies/mL HIV RNA (interquartile range (IQR) 7.26 E4). The median CD4$^+$ T-cell count at time of sampling was 422 CD4$^+$ (IQR 439) and the median nadir CD4$^+$ T-cell count was 310 (IQR 261) cells/mm^3. Neither low CD4$^+$ cell-count at time of sampling (p 0.636) nor low nadir CD4$^+$ count (p 0.462) was associated with either X4 or R5 prediction. Tropism was predicted using two interpretation algorithms; G2P and PSSM$_{X4\text{-}R5}$. Using an FPR of 10% the number of X4-predicted sequences did not significantly differ between G2P and PSSM$_{X4\text{-}R5}$ (p 0.186), 28 sequences were predicted to be X4-tropic in G2P and R5-tropic in PSSM$_{X4\text{-}R5}$. Conversely, 15 sequences were predicted to be R5-tropic in G2P and X4-tropic in PSSM$_{X4\text{-}R5}$. As G2P is the most commonly used interpretation algorithm in clinical practice in Europe further analysis was performed with G2P only.

Using a triplicate procedure a total of 266 (87.8%) sequences were generated (156 from 58 viral RNA samples and 110 from 43 proviral DNA samples) (Fig. 1). Amplification failures were observed in 14% of proviral DNA samples and in 10% of viral RNA samples. Amplification failures were not specifically associated with low viral RNA load (p 0.249). Median viral RNA load of samples with an amplification failure was 7.9 E3 (IQR 1.221 E5) compared with 8.7 E3 (IQR 5.479 E4) for samples with no amplification failure.

The majority of sequences had an FPR above 10% (*n* = 202, 75.9%). After dividing the data into 10% FPR increments, these sequences are distributed throughout the different categories. The remaining 24.1% (*n* = 64) fall into the 0–10% FPR increment, which therefore was the largest category (Fig. 2). Using single genotypic tropism testing successful tropism results were generated for 92.1% of the samples. The success rate increased into 100% tropism results when tested in duplicate and triplicate. Pooled analysis of triplicate

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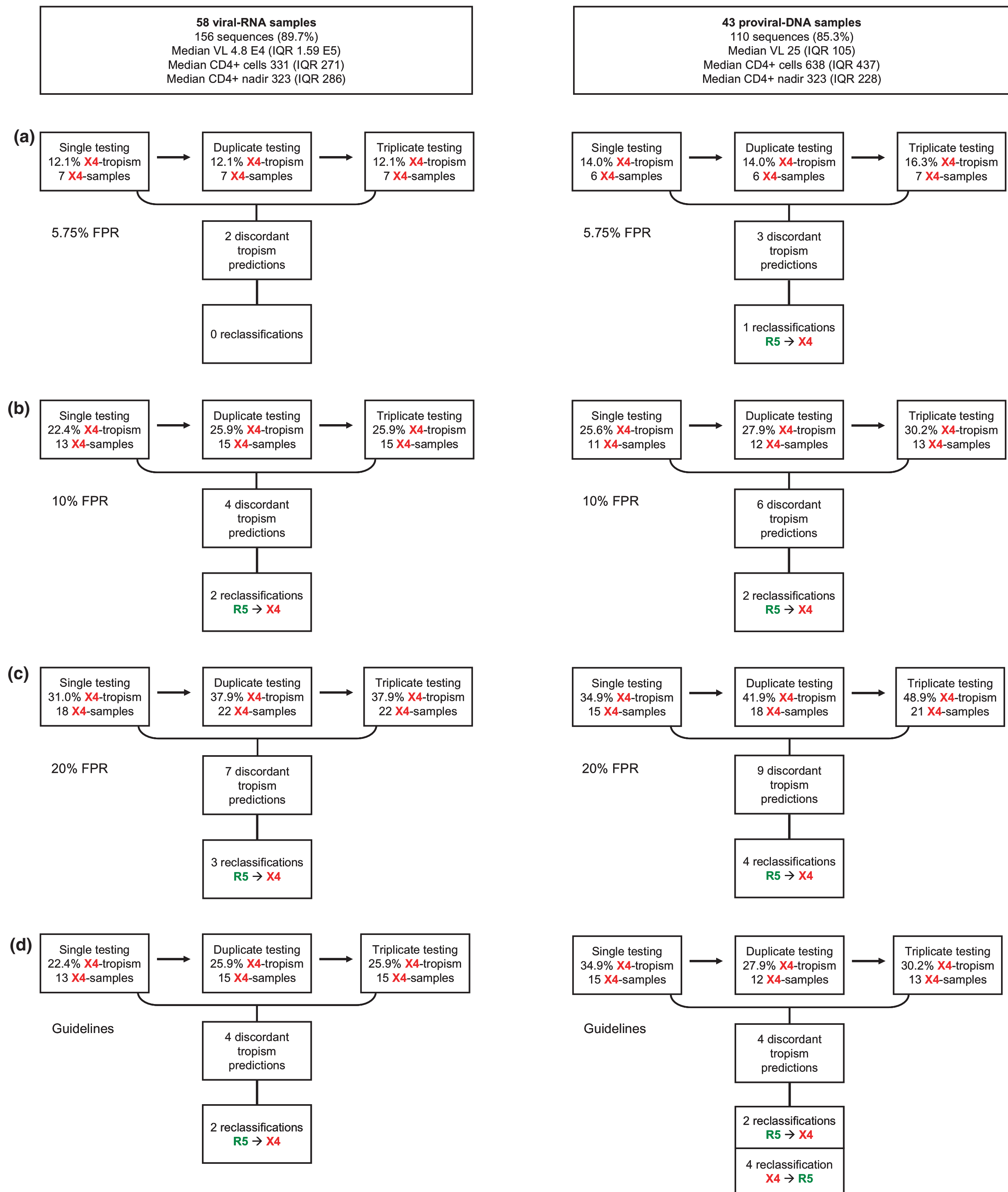

FIG. 1. A total of 101 patient samples tested in a single versus triplicate genotypic tropism procedure (58 viral RNA samples and 43 proviral DNA samples). VL = HIV-1 RNA level copies/mL, number of successfully analysed sequences are listed. Tropism was predicted using a false-positive rate (FPR) of 5.75% (a), 10% (b), 20% (c) or according to the current European guidelines (d) [7]. Percentage X4-predicted samples is depicted.

sequence data from individual patient samples resulted in an X4 prediction in 25.9% of the viral RNA samples and 30.2% of the viral DNA samples (FPR 10%; p 0.628) (Fig. 1a).

Samples for which one of the sequences resulted in an R5 prediction while at least one of the other sequences yielded X4 results, were considered discordant. Analysis of the data

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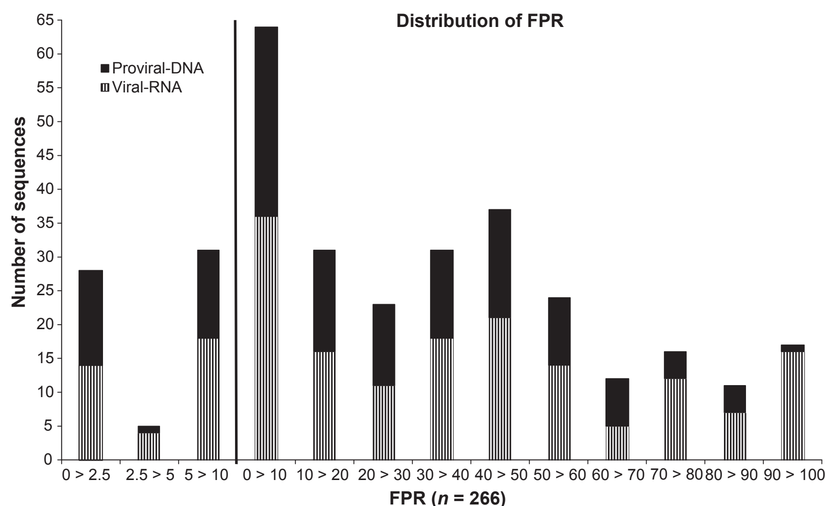

FIG. 2. Distribution of Geno2Pheno$_{[coreceptor]}$ results of 266 sequences obtained from 101 patient samples. Black bars represent proviral DNA sequences, striped bars represent viral RNA sequences. Distribution of 0>10 false-positive rate (FPR) is also subdivided in categories 0>2.5, 2.5>5 and 5>10.

TABLE 1. Overview of reclassified samples using a triplicate tropism procedure. X4 prediction is given in red and R5 prediction in green, – indicates amplification failure and NA = not assessed. Guidelines: European guidelines on clinical management of HIV-1 tropism testing [7]

FPR	Sample id	Sample	Subtype	Single FPR	Triplicate procedure FPR 1st	FPR 2nd	FPR 3rd	Viral load	CD4 count	CD4 nadir	Reclassification
5.75%	A	*DNA*	B	8.2	8.2	-	4.1	20	669	NA	R5 → X4
10%	B	RNA	CRF01_AE	33.0	33.0	7.4	-	48100	5	5	R5 → X4
↓	C	RNA	B	16.9	16.9	6.6	-	74651	467	467	R5 → X4
	D	*DNA*	B	53.5	53.5	17.9	7.8	25	1085	635	R5 → X4
	E	*DNA*	B	55.8	55.8	8.7	17.0	130	658	378	R5 → X4
20%	B	RNA	CRF01_AE	33.0	33.0	7.4	-	48100	5	5	R5 → X4
↓	F	RNA	AB	20.1	20.1	18.3	-	30000	NA	NA	R5 → X4
	G	RNA	B	33.7	33.7	17.0	33.7	648	318	281	R5 → X4
	D	*DNA*	B	53.5	53.5	17.9	7.8	25	1085	635	R5 → X4
	E	*DNA*	B	55.8	55.8	8.7	17.0	130	658	378	R5 → X4
	H	*DNA*	B	34.8	34.8	32.6	16.9	25	621	283	R5 → X4
	I	*DNA*	B	49.9	49.9	24.7	13.7	25	761	278	R5 → X4
Guidelines*	B	RNA	CRF01_AE	33.0	33.0	7.4	-	48100	5	5	R5 → X4
↓	C	RNA	B	16.9	16.9	6.6	-	74651	467	467	R5 → X4
	D	*DNA*	B	53.5	53.5	17.9	7.8	25	1085	635	R5 → X4
	E	*DNA*	B	55.8	55.8	8.7	17.0	130	658	378	R5 → X4
	J	*DNA*	B	16.9	16.9	49.7	19.6	600	458	378	X4 → R5
	K	*DNA*	B	14.7	14.7	20.8	-	130	245	NA	X4 → R5
	L	*DNA*	B	17.0	17.0	-	15.7	25	750	413	X4 → R5
	M	*DNA*	B	13.7	13.7	15.6	12.8	25	890	4	X4 → R5

[a]The European guidelines advise a false-positive rate (FPR) of 10% for triplicate procedures. For a single procedure an FPR of 20% for RNA samples with a viral load <1000 copies/mL and for proviral DNA samples is advised.

with an FPR of 10% resulted in ten discordant tropism results, four viral RNA and six proviral DNA samples (9.9%) had discordant tropism results (Fig. 1).

Discordance does not always result in reclassification of the tropism report. If a population is predicted to be X4-tropic in the first replicate, finding an R5-tropic virus in the second or third replicate will not change the tropism prediction.

Therefore we analysed for each individual patient sample the influence of triplicate testing on the reported tropism result. If only the first replicate was taken into account, 24 samples (both RNA and DNA samples) were predicted to

be X4-tropic (23.8%). Adding the second replicate increased the number of X4-predicted samples to 27 (26.7%) and addition of the third replicate resulted in 28 samples with X4 tropism results (27.7%) (Fig. 1). Hence, triplicate testing using an FPR of 10% resulted in a 16.7% increase of X4-predicted samples. This corresponds to a 4% increase in X4 prediction for the total study population (p 0.730) and reclassification from R5 to X4 tropism in four patients (RNA $n = 2$; DNA $n = 2$) (Table 1). Additionally we analysed the data with three random sets of first replicates using an FPR of 10%. These three data sets resulted in ten discordant samples with four, five or eight reclassifications from R5 to X4 tropism, respectively (p ≥0.278). Randomizing the order of replicates did not influence our results.

Triplicate analyses using a more conservative 20% FPR increased the total number of discordant samples to 16 (15.8%) (RNA $n = 7$; DNA $n = 9$) (Fig. 1) and the number of reclassifications from R5 to X4 tropism to seven (6.9%, p 0.353) (Table 1). Analysis of the sequence replicates in a different order did not significantly change the level of reclassifications (all p values ≥0.310). The number of discordant samples did not significantly differ in proviral DNA samples (low viral load) or RNA samples (high viral load) (p ≥0.228 depending on FPR).

We also analysed the samples according to the European guidelines for clinical management of HIV-1 tropism testing [7]. In this analysis the first replicate resulted in 28 (27.7%) samples predicted to be X4-tropic. Triplicate genotypic tropism testing did not change the overall number of X4-predicted samples (Fig. 1). However, this analysis resulted in eight discordant samples (RNA $n = 4$, DNA $n = 4$). Furthermore, two viral RNA samples and two proviral DNA samples were reclassified from R5-tropic to X4-tropic and four proviral DNA samples were reclassified from X4-tropic to R5-tropic (Table 1). In literature, a low FPR of 5.75% was found to be a good predictor for response to MVC treatment in phenotypically pre-screened patients [20]. In general, application of a lower FPR results in less frequent prediction of X4 virus and therefore a decreased level of discordance. In our data set triplicate testing with an FPR of 5.75% yielded five discordant samples (RNA $n = 2$, DNA $n = 3$) resulting in reclassification from R5 to X4 tropism in only one patient (p 0.995) (Fig. 1).

Discussion

We evaluated the added value of triplicate versus single testing on genotypic tropism prediction in routine clinical practice. Co-receptor usage of virus isolates from 101 patient samples was predicted after V3 sequencing and applying publicly available and commonly used interpretation algorithms. In this study, patient samples with a broad range of HIV RNA plasma levels as well as proviral DNA samples were analysed with G2P with an FPR of 5.75%, 10%, 20% and according to current European guidelines on tropism testing.

A considerable number of samples with discordant triplicate results was observed. In one out of every ten samples X4-predicted as well as R5-predicted sequences were detected. However, in only half of these cases did the discordance result in a reclassification of the final tropism call from R5 to X4.

One could argue that in samples with a low viral input the observed discordance in the triplicate analyses results from stochastic errors in sampling and amplification. However, the number of discordant results did not significantly differ between samples with low or high viral load. Therefore it seems more likely that the overall high levels of variation in env is the reason for the observed discordance in the triplicate analysis of viral tropism. Detection of nucleotide mixtures in a sequence complicates the tropism prediction. In the G2P algorithm nucleotide sequences are used as input and amino acid mixtures are therefore considered. In $PSSM_{X4\text{-}R5}$ amino acid sequences are used as input and all possible amino acid combinations have to be considered manually. Taking mixtures into account may overcall X4 prediction because not every combination may actually be present in the viral population. Next generation, ultra-deep pyrosequencing may partly solve this issue because every strain is separately sequenced using this technique. Our results could not be compared with ESTA because almost half of our samples had a viral load below the minimum requirement of 1000 copies/mL for European samples.

Our study is the first that compares single with triplicate genotypic tropism testing in clinical practice. A recent study investigated the added value of tropism determination using duplicate PCR amplification and pooled sequencing. All possible amino acid sequences of the V3 loop were interpreted with G2P using an FPR of 10% [21]. The number of X4 tropism results in this particular duplicate approach increased from 25 to 30 (3.3%), which is in line with our observations.

On the individual patient level, our triplicate procedure increased the detection of X4 variants, thereby decreasing the number of patients eligible for MVC treatment. Unfortunately we cannot present clinical outcome data comparing single with triplicate testing because our study was designed as a prospective analysis and triplicate testing results were part of the clinical decision-making. Triplicate testing may have implications for the efficacy of MVC-containing therapy. Failure to detect an X4 virus in a single genotypic procedure

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may lead to selection of X4 virus, MVC therapy failure and loss of backbone activity [22].

In conclusion, independent of the applied FPR, triplicate testing increased X4 prediction in individual cases. Our results illustrate that comparison of single with triplicate amplification procedures in relation to clinical outcome data is urgently needed. Pending these data, we prefer to be conservative and increase the sensitivity of genotypic tropism testing by performing a triplicate procedure in routine clinical practice.

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Transparency Declaration

There are no conflicts of interest to declare.

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