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High-risk sexual practices contribute to HIV-1 double infection among MSM in Madrid.

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Running head: HIV-1 DI prevalence in Madrid MSM

Keywords: HIV double infection, Prevalence, MSM, Madrid, Risk practices, Sexual Partners, Viral genetic variability.

ABSTRACT:

Background: Data on the prevalence of double infection (DI) in HIV individuals are lacking in Spain. To fill this gap, we analyzed the prevalence of DI in a cohort of men who have sex with men (MSM) and examined factors contributing to DI.

Methods: We selected 81 MSM attending Centro Sanitario Sandoval, a Sexually Transmitted Diseases Clinic in Madrid. We obtained by ultra-deep sequencing the proviral sequences in *gag* and *env* genes and performed a phylogenetic analysis for the identification of DI. Clinical, behavioral, host and viral factors were studied for its association with DI.

Results: We detected 6 individuals with DI and one case of superinfection with a global prevalence of 8.6%. The genetic distance among the subtype B viruses in mono-infected individuals (24.4%) was lower than the distance between the two viruses in subtype B DI individuals (29.5%). Individuals with a high number of sexual contacts (>25 partners/year) had an 8.66 times higher risk of DI (p=0.017).

Conclusions: In this MSM cohort the prevalence of HIV DI was estimated at 8.6%. DI was strongly associated with the number of sexual partners. Because of the pathogenic consequences of HIV DI, this high prevalence should promote Public Health Programs targeted to high risk population such as MSM for the control of HIV infection and DI. HIV DI should be considered for a better clinical management of these individuals.

Words: 228

INTRODUCTION

HIV patients could be infected by more than one heterologous viral strain. This phenomenon named dual infection (DI) could take place as co-infection (CI), when both virus are acquired simultaneously or at least before seroconversion, or as superinfection (SI) when the infection with the second strain occurs after seroconversion.

DI was first reported in a single case[1] and in multiple descriptions of individual cases, reviewed in[2]. DI prevalence rates in cohort studies with distinct designs and risk populations in different countries have been also reported.[2,3]

Currently, HIV epidemic in Spain is driven mostly by the group of men who have sex with men (MSM) in which the incidence has risen from 47.5% in 2009 to 56% in 2018. MSM accounts for the majority (56.4%) of the 3,244 new diagnosed cases in 2018.[4] Epidemiological studies showed that MSM engage in high risk practices, increasing the risk of sexually transmitted diseases (STD), including mono a double HIV infection.[5,6]

Previous works revealed that DI prevalence varies with the type of transmission. It may reach 50% in intravenous drug users (IDU), while in heterosexual individuals prevalence is usually lower.[7,8] In MSM, the DI prevalence was estimated at 14.4% in San Diego (CA, USA),[9] 12.2% in Brazil,[10] 12.3 - 13.3% in China[11,12] and 6.8% in Belgium.[13]

Studies suggested that the human leukocyte antigen alleles may play a role in HIV-1 DI, like the HLA-B35 that was over-represented in these patients.[9,14] This and other alleles such as the HLA-A29, HLA-C16 and HLADRB1-07 were associated with SI.[15]

Most works proposed an impact of DI in clinical progression markers, like increase in viral load (VL),[9,16,17] accelerated decline in CD4+T cells, shortened time to AIDS[11,18] and HIV-1-associated neurocognitive disorders.[19] DI could compromise HIV antiretroviral treatment because of recombination between resistant genomes.[20,21] However, there are unexpected increases in VL not associated with DI,[22] and our group described the maintenance of the clinical status in DI long term non-progressors (LTNPs).[14,23]

To estimate the prevalence of HIV-1 DI in MSM in Madrid and the potential contributing factors, we established a cohort of 81 individuals followed in a primary Sexual Transmitted Diseases Clinic. We analyzed by phylogenetic methods the individual´s proviral sequences in *gag* and *env* genes obtained by Ultra-deep sequencing (UDS). In addition, we analyzed the association of different virological, epidemiological and behavioral factors with HIV DI.

MATERIALS AND METHODS

Study population

To determine the prevalence of DI in Madrid (Spain) and contributing factors, we selected study participants from patients followed at the Centro Sanitario Sandoval (Hospital Clínico San Carlos, IdISSC, Madrid, Spain). This is a monographic, outpatient clinic, oriented to prevention, diagnosis and treatment

of Sexually Transmitted Infections (STDs). Among its healthcare characteristics is the great accessibility (patients do not need an appointment or a health card), rapid and conventional microbiological diagnosis, immediate administration of STD treatment, and close interaction with Primary Care, Hospitals and NGOs. These characteristics have facilitated the recruitment of populations that are especially exposed to STDs and have made the clinic a sentinel device for the epidemiological surveillance of STD/HIV in Madrid and a reference in Spain. In the last year 5300 MSM patients were attended for HIV testing and 320 HIV-1 positive patients for STD monitoring.

We performed a prospective cohort study in 81 HIV-1 positive MSM. Inclusion criteria were MSM with risk practices such as sexual contacts with lack of systematic condom use and STDs in the follow-up, CD4+ T-cell levels above 500 cell/μl and naïve for antiretroviral treatment. 26 individuals were enrolled within the first year of HIV diagnosis, 23 between the second and the third year and in 26 after more than 3 years. In the remaining 6 patients the date of the diagnosis is missing (Supplementary Data (SD) Table 1). The study was planned to follow-up the patients for 30 months (from January 2013 to June 2016), collecting blood samples and clinical data every 6 months. Finally, we followed 51 patients (63%) for a median of 12 months [interquartile range (IQR): 8-15 months] (SD Table 1). At enrolment and during the follow-up all patients answered to a structured epidemiological questionnaire validated by Centro Sanitario Sandoval in order to determine their socio-demographic characteristics, sexual practices, frequency of condom use, history of STDs throughout their sexual lives, blood donations, toxic habits and clinical markers. These data are summarized in Table 1.

All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all participants. The study was approved by the Comité de Ética de la Investigación y de Bienestar Animal of the Instituto de Salud Carlos III with CEI PI 32_2013-v4 reference number.

Clinical characteristics of the patients

All HIV-1 cases were diagnosed by the chemiluminescent microparticle immunoassay (CMIA,) ARCHITECT HIV Ag/Ab Combo assay (Abbott, Spain) and Bio-Rad Geenius HIV 1/2 Confirmatory Assay (Bio-Rad, Spain). Viral loads were determined by Versant® HIV-1 RNA 1.5 assay (kPCR) using Versant kPCR molecular system (Siemens, Spain). CD4+ and CD8+ T-cell counts were measured by flow cytometry (Epics® XL Beckman Coulter).

STDs diagnosis included the detection of primary or secondary Syphilis (by microscopy and serology), Neisseria gonorrhea (by Gram staining and growth in Thayer Martin medium) and Chlamydia trachomatis (CT) performed by PCR. The ompA and pmpH genes were sequenced in those CT positive samples for Lymphogranuloma venereum (LGV) genotypes. Diagnosis was undertaken from urethral, pharyngeal, cervical and rectal samples.

DNA extraction and proviral HIV-1 DNA quantification

Peripheral blood mononuclear cells (PBMC) and plasma samples were collected at the enrollment and at each follow-up visit. Proviral DNA was obtained from 5 x 10^6 PBMCs using the Speedtools tissue DNA extraction kit (Biotools B&M Labs S.A., Spain). DNA quantification was performed by real-time PCR protocol as described in[24] with the following modifications: in the first

PCR we used 1x NH4 reaction buffer (Bioline, Spain) and BIOTAQ DNA polymerase; the second PCR was performed with a LightCycler instrument (Roche, Spain) using the LightCycler Taqman Master Mix (Roche, Spain); normalization was performed by amplification of glyceraldehyde phosphate dehydrogenase (GAPDH) gene in a separate PCR reaction using the primers indicated in SD Table 2 and the Universal Probe Library probe #64 (Roche, Spain). The detection limit was established at 10 copies/1.67 x 10^5 PBMC based on the results obtained with a standard curve generated with serial dilutions of ACH2 cells (1 HIV-1 copy per cell) in healthy donor PBMCS – DNA.

MiSeq library preparation and sequencing

To ensure the detection of HIV-1 minority variants in each patient, at least 200 copies of proviral DNA were used for Ultra Deep Sequencing (UDS) performed in the Illumina MiSeq platform (San Diego, CA, USA). Two regions within HIV-1 *gag* (423 base pairs) and *env* (391 base pairs) genes were obtained from all available PBMC samples. First PCR was performed with Phusion High-Fidelity DNA Polymerase (Thermo Fisher, Spain), two sense and two antisense primers (SD Table 2) and a hybridization temperature of 62ºC, for 35 cycles. The resulting amplicons were used as template for two second PCRs with specific primers in each gene for the incorporation of Illumina indexing adaptors (SD Table 2) in the same conditions than in the first PCR. A dual-index sequencing strategy was used to minimize the number of primers needed for multiplex run. Briefly, 8-bp-long indexes (or “barcodes”) were added to the 5’ and 3’ ends of each fragment. A unique pair of indexes was used for each sample (http://dx.doi.org/10.1128/AEM.01043-13). Eight i5 forward and twenty-four i7 reverse indices were used (Illumina Nextera XT v2 index sets A and B). Index

tags were added in a third PCR (15 cycles), as recommended (Illumina Nextera XT). Indexed MiSeq amplicons were quantified using the QuantiFluor® ONE dsDNA System (Promega, Spain) and pooled at equimolar concentrations prior to MiSeq sequencing with a 2x250bp v2 kit. All PCRs amplifications were done with procedural safeguards, physical separation of sample processing and post-PCR handling steps to control cross-contamination.

Characterization of genetic markers of the patients

HLA genotyping of HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 alleles was performed, from genomic DNA, in the DI patients and in a sub-population of 22 mono-infected individuals (MI, 3 times the number of HIV-1 DI patients). MI patients of this group were selected with the same distribution of age, origin and infection time than in the initial cohort. The analysis was carried out using a PCR-SSOP hybridization system linked to Luminex detection (Immucor, USA).[25] Because of the limited number of individuals and the power of the study, only two-digit HLA lineage codes were used.

Ultra-Deep Sequencing Analysis (UDS)

UDS sequencing data were analyzed with a home-developed pipeline where FASTQ sequence files were processed via a multi-step method. Raw sequences, were filtered with TRIMMOMATIC,[26] defining fragments of 250 base pairs (bp) with a minimum length of 220 bp and a 30:20 quality threshold (median 50,002 per sample, range: 11,924 – 244,735). Resulting paired-end sequences were merged into single sequences using PEAR software (median 46,759 per sample, range 11,802 – 236.495).[27] Usearch was used to convert Fastq file to Fasta format.[28] Extracted sequences from each HIV-1 gene *gag*

(median 18,351, range 0 – 156,627) and *env* (median 25,330 per sample, range 2,946 – 174,393) were clustered into Operational Taxonomic Units (OTUs) with a 97% similarity threshold after removing all singleton sequences (https://www.drive5.com/usearch/manual/otus.html). The 97% similarity threshold was determined with a control sample. In this control assay, 200 copies of the 89ES061 HIV-1 molecular clone[29] diluted in DNA from healthy donor PBMCs were amplified and sequenced as described. The raw sequences acquired were used to generate OTUS at 99.9%, 99.5%, 99.3%, 99%, 98% and 97% identity. Only the 97% similarity threshold correctly identified one single OTUs. Phylogenetic trees with the OTUs obtained in this work and other nucleotide sequences recorded in the laboratory were generated to identify cross-contaminating sequences. Finally, the phylogenetic trees in *env* and *gag* genes were inferred using the Neighbor-Joining method in MEGA 6.[30] The evolutionary distances were computed using the Maximum Composite Likelihood method, the variation rates among sites were modeled with a gamma distribution (shape parameter = 0.805 for *env* and 0.461 for *gag* gene) and bootstrapping (with 1000 replications) tested the branch support.

WebPSSM bioinformatic tool[31] was used to predicte HIV-1 coreceptor usage from the amino acid sequence of the third variable loop (V3) of the envelope gene. Virus subtyping was done using COMET (Context-based Modeling for Expeditious Typing) HIV-1 (Version 1.0)[32] and the Rega Subtiping Tool (Version 3.0)[33] using default reference nucleotide sequences sets.

Single genomes analysis

To confirm the results obtained by UDS in some DI cases, single-genome amplifications followed by Sanger sequencing, as described in,[34] were performed in proviral DNA in *gag* and *env* genes with the same primers used for UDS.

Definition of HIV-1 Dual Infection and Superinfection

DI was phylogenetically identified when individual´s viral sequences (represented by OTUs), displayed a genetic distance between them higher than 5% and grouped in two different cluster (with a high bootstrap value >95%). HIV-1 superinfection was determined when in the first sample viral sequences formed a monophyletic cluster, but in the later time points they grouped in two or more divergent clusters.

Statistical analysis

Variables were summarized as medians and interquartile ranges (IQR) when continuous, and as percentages when categorical. Differences in patients' characteristics according to DI status were evaluated with Fisher test (nominal variables) and Kruskal-Wallis test (continuous variables). Odds ratios (OR) for the association between patients' characteristics and DI status were estimated with logistic regression models. To test the risk of DI in a given HLA allele carrier vs non-carrier, since data were sparse, OR were estimated with small sample adjustment and their confidence interval calculated with normal approximation with small sample adjustment using the *epitools* package in R. The distribution of HLA alleles was compared between MI and DI participants. Relative risk ratios (RRR) for the association between HLA specific alleles and

MI and DI status were estimated with multinomial Firth logistic regression.[35] All statistical analyses were performed using R version 3.4.3 (R Core Team, 2017).

RESULTS

DI prevalence in the MSM cohort

DI cases were identified by the presence of viral sequence in different branches of the tree supported by high bootstrap values (>95%) in *env* (Figure 1a) and *gag* genes (Figure 2). All the DI cases were detected in *env* gene and in only 5 patients in *gag* region (Figure 1a, Figure 2 and Table 2). Because of the low representation in the viral population of one of the two viruses in DI 03, DI 08, DI 52 and DI 56 patients´ samples, we performed single-genome nucleotide sequence analysis. This approach confirmed the presence of the two viruses (Table 2). In the first sample analyzed, 6 individuals showed DI; 4 of them were intra-subtype B DI, and 2 cases of inter-subtype DI with B/F1 and B/CRF 02 AG viruses (Figure 1a and Table 2). In patient DI 03, only one virus was detected in the first sample, however during the follow-up two subtype B viruses were identified in both genes indicating SI (Figure 1a and Table 2). Overall, a DI prevalence of 8.6%, with 6 cases of DI and 1 SI was recognized in this group.

Genetic variability of the patient's samples

Within all the patients analyzed, 87.5% were infected with subtype B viruses, 8% with CRFs, 3.4% with subtype C and 1.1% with subtype F1 viruses (SD Table 3). Sequences from the OTUs were also analyzed for phenotypic

characteristics, and only 10 patients showed minority CXCR4 phenotype sequences, including 3 sequences in one of the viruses from patient DI 08 (Table 2).

Non subtype B viruses prevalence was similar between MI (12.2%) and DI (14.3%) patients. We also characterized the diversity of the viruses, analyzing the mean genetic distance among all subtype B sequences from MI patients. Median genetic distances among all MI individuals' subtype B sequences was 24.2% (mean = 24.4%) (Figure 1b). The median genetic distance between the two subtype B viruses in DI cases was 29.1% (mean 29.5%). This difference was statistically significant (p=0.035)**.** This difference in median genetic distance is maintained and even increased when considering all viruses, regardless of HIV subtype.

Analysis of potential HLA alleles associated with DI

Host genetic data were available for 29 participants, all DI patients and 22 control MI selected individuals. We compared the frequency of HLA-A, HLA-B, HLA-C, HLA-DQB1 and HLA-DRB1 alleles between the MI patients and the DI patients (SD Table 4). Since data were sparse, OR were estimated with small sample adjustment and their confidence interval calculated with normal approximation with small sample adjustment. Risk of DI was higher (only at a $p<0.1$ level of significance) for HLA-DQB1*04 (p=0.052), HLA-DRB1*08 (p= 0.052), HLA-C*07 (p= 0.092) and HLA-C*12 (p= 0.075).

Clinical and epidemiological characteristics of the cohort individuals.

The characteristics of the 81 cohort individuals overall and stratified by MI (N=74) and DI (N=7) status are presented in Table 1. The intervals selected to

analyze the continuous variables were based on the previous experience of the Centro Sanitario Sandoval [36]. Participants with DI came more frequently from other countries than Spain and had been infected for longer time than MI participants, although the differences were not statistically significant (Table 1). DI patients tent to have slightly more STD than MI patients. 86% of the DI individuals had STDs previous to HIV-1 diagnosis, in contrast with only 70% of the MI patients (Table 1). When we consider the number of STDs, 71% of the DI persons showed more than 3 episodes, whereas in MI patients this occurred in only 40% (Table 1).

Regarding number of SP, 85% individuals with DI reported having more than 25 SP in the last year versus the 37.8% of MI (p=0.017) (see Table 1). Similar results were observed when the number of SP were quantified during the lifetime although the association is borderline significant (p=0.098). The plasma viral load copies, the number of CD4+ cells (Table 1), the CD8+ cells and the CD4/CD8 ratios (data not shown) were similar in the two groups.

In terms of associations, individuals who reported more than 25 sexual partners during the last year had a risk 8.66 times higher of being DI than those with less than 25 SP (p=0.021, Table 3). After further adjustment for age, the OR increased up to 11.8 (95%CI: 1.24, 111.6) and remained statistically significant (p-value= 0.031). Similar finding was obtained after further adjustment for viral load, CD4+ and CD4+/CD8+ ratio [OR=11.2 (95%CI: 1.07, 117.7), p-value= 0.044)].

DISCUSSION.

Although DI in Spanish patients has been previously described,[14,37-39] there are no specific studies on DI prevalence in MSM cohorts who nowadays contribute the most to the epidemic in Spain. In this work, analyzing two genes by UDS, we detected 7 individuals with DI in a cohort of 81 MSM in Madrid, estimating a DI prevalence of 8.6%. One of DI was a confirmed case of SI, which imply a SI incidence of 2% (1/51) during the study period.

The DI prevalence in this cohort (8.6%) was similar to the prevalence in MSM in Belgium (6.8%)[13] but lower than the prevalence (14.4%) observed in San Diego[19] or in China (12.3% - 13.3%).[11,12] Discrepancies can be attributed to the different methods, sample or time since seroconversion used to detect them. For the DI detection, it is convenient to obtain samples close to the DI event, because recombinant strains could arise making difficult the identification of the two strains by phylogenetic analysis.[19,39,40] Moreover, the different viruses are not expressed in the same proportion along the infection and they could be lost or not detected.[6,8,13,19,41,42] Recombination may be the reason why were not able to detect DI in *gag* gene in two patients (Table 2).

In our study, SI was detected in only one out the 51 individuals (2%) during the follow-up which is lower than the SI incidence observed in[12,13,15]. This difference could be due in part by our sampling schedule. In our work samples were taken on average 28 months after diagnosis (SD Table 1) whereas in the other studies, samples were analyzed during the first months or year of infection.[12,13,15] Because of the long time elapsed since diagnosis, we could not discern between CI and SI in the DI cases, and this could really underestimate the true SI incidence in our cohort, as many of the DI cases could be in fact due to SI.

In this study, we found a higher genetic distance between the two subtype B viruses in the DI patients than among the subtype B viruses circulating in the MI patients (Figure 1b). The wide divergence between the viruses present in DI patients would be even greater if we consider the genetic distance between viruses in the inter-subtype DI (patients DI 16 and DI 56, blue lines Figure 1b) The higher genetic distance found between DI viruses could indicate a potential cross immunological protection after HIV primary infection. It is possible that the immune response produced by the first infection is able to protect against similar strains but not against a second strain that has a high antigenic divergence from the first virus. High genetic diversity has been related to the failure of HIV vaccination programs and also linked with pathogenicity.[43]

DI has been associated with different markers of disease progression such as increase in the VL, high CD4+ cells decline and rapid disease progression.[9,11,16-18] In addition, the presence of two viruses in the individuals is prone to the generation of recombinant viruses: these new variants could have pathogenic consequences if, for example, two resistant genomes could recombine to form a virus with extended resistance.[20] In this study, we did not find any statistical difference between different biomarkers (VL, CD4+ T cell number) between MI and DI patients (Table 1). This is probably based in the short follow-up of the patients (SD Table 1).

Some HLA alleles were associated with an increased risk of DI (HLA-DQB1*04, HLA-DRB1*08, HLA-C*07 and HLA-C*12), although at a $p<0.1$ level of significance. The DRB1*08 allele has been already associated with an increased risk of SI.[15] Although HLA-DQB1*04 and HLA_DRB1*08 are more prevalent in Central and South America, the relationship found in this work does

not appear to be due to the origin of the patients. Both alleles appear linked together in two DI patients, one from Spain and the other from Argentina. These results are based on a small sample size of DI cases, are not adjusted for multiple testing and thus should be taken with caution. The possible role of these HLA alleles on DI should be analyzed in larger cohorts.

Persons living with HIV who continue to practice unprotected intercourses have increased risk not only for new STDs, but also for HIV reinfection. As previously stated, STDs favor HIV infection.[5] The analysis of STDs in our cohort showed that DI persons had more records of STDs and with more episodes of these infections, although these results did not reach a statistical value. This result is plausible since most of the people engaged in this study attend this STD clinic primarily because of STDs suspicion.

The main result of the study is the strong association of DI with the number of SP that was established at above 25 partners/year (Table 3). Similar association was also suggested in a previous study although established a lower level of partners.[15] The association of SP and DI was found, even though, all participants included in this cohort showed high-risk practices (sexual contacts without systematic condom use and with STDs in the follow-up).

CONCLUSIONS

An HIV DI prevalence of 8.6% was found in this MSM cohort. DI was strongly associated with the number of sexual partners. The data of this study support the need for prevention programs targeted to MSM populations in order to control not only HIV infection, but also DI. Preventive programs should focus

both on the prevention of HIV but also on other STDs. Finally, because of the potential pathogenic consequences of HIV, DI should be considered for the clinical follow-up and for the early initiation of antiviral therapy.

DECLARATIONS

Ethical Approval and consent to participate

All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki and written informed consent was obtained from all participants. The work has been approved by the Comité de Ética de la Investigación y de Bienestar Animal of the Instituto de Salud Carlos III (CEI PI 32_2013-v4 number).

Consent for publication

Not applicable.

Competing Interest.

None of the authors declared conflict of interest related to this work.

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Authors' contributions

CC, MP, IO, CR, MV, JDR and CLG contributed to the study design, data interpretation and performed data analysis. MR designed and performed all the epidemiological and statistical analysis. OA, MV, CR and JDR carried out the selection, clinical follow-up of the patients and the analysis of Clinical Data. RA and JLV performed the HLA typing and analysis. PJ and AZ carried out the UDS work and helped in the sequence analysis. CC, MP, MR, JLV, AZ and CLG wrote the manuscript.

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