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Determinants of HIV-1 Mother-to-Child transmission in Southern Brazil

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ABSTRACT

Different human immunodeficiency virus type 1 (HIV-1) subtypes may have distinct biological, immunological and pathogenic properties. Efficiency of mother-to-child transmission (MTCT) may be among those properties, but few and controversial results have been described so far. In this study, 102 children born from HIV-1-infected mothers between 1998 and 2004 in the city of Rio Grande, Brazil were analyzed for potential risk factors associated with MTCT. That geographic region is characterized by a high proportion of subtype C-infected subjects, and it allowed comparison between subtypes B and C and their influence on MTCT. The analysis also included clinical, obstetric and immunological parameters. Multivariate regression analyses were conducted to evaluate the influence of the parameters on MTCT, and prevalence ratios (PR) and 95% confidence intervals (CI95) were also calculated. A surprisingly high prevalence of subtype C of over 70% was found. Only the HIV viral load and the use of ACTG 076 protocol were predictive of MTCT. HIV subtype and CD4 T-cell counts were not associated with increased risk of transmission. Although a clear expansion of subtype C is evident in southern Brazil, it does not seem to correlate with increased risk of vertical transmission.

Key words: HIV, MTCT, vertical transmission, subtype C, molecular epidemiology.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) can be divided in three major groups based on its genetic variability: M or main, O or outlier and N or new. Whereas groups O and N are of low prevalence and are restricted to certain areas of the African continent, group M is responsible for the AIDS pandemic (Peeters 2001). Spreading out of Africa, group M viruses have evolved and dispersed in different lineages known as subtypes. Currently, nine subtypes and at least 16 recombinant circulating forms (CRFs) are distinctly recognized (Perrin et al. 2003), with distinct and dynamic prevalence distributions in different parts of the world. It is not known, however, whether those differences simply reflect historical and founder effects of the several HIV-1 subtypes, or if the dynamics of subtype-

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specific transmission potentials play a significant role (Kanki et al. 1999). An important example of such changes in HIV-1 subtype distributions includes the significant expansion of subtype C in southern and southeastern Africa, India and China (Novitsky et al. 2002). More than 90% of new HIV-1 infections take place in Africa and Asia, where the epidemics is mainly driven by subtype C, and where over 25% of adults are infected in some of these countries (Essex 1999). Currently, subtype C is responsible for over 60% of the HIV-1 infections (Esparza and Bhamarapravati 2000).

In Brazil, the major circulating HIV-1 subtype is B, although subtypes C, D and F as well as diverse mosaic genomes have been described (Morgado et al. 1998, Martínez et al. 2002, Brindeiro et al. 2003, Soares et al. 2003). The city of Rio Grande, RS, situated in the extreme south of Brazil, is a harbor city with a great number of human interflows to and from Africa, Asia and Europe. In this sense, the role of immigrants in the introduction and prevalence of non-B subtypes of HIV-1 may be paramount (Perrin et al. 2003). In Rio Grande, although subtype B still predominates, subtype C was already responsible for 22% of the AIDS cases in 1997 (Martínez et al. 2002).

The biological and epidemiological implications of HIV-1 genetic variability remain to be elucidated. Some reports have suggested that different subtypes may have distinct biological features that would impact on the transmissibility and on disease progression in infected patients. CD4 T-cell counts were lower and HIV viral loads were higher in subtype C-infected than in subtype B-infected individuals (Neilson et al. 1999). Some reports have also tried to characterize maternal factors that may affect HIV-1 mother to child transmission (MTCT) in African pregnant women (Bobat et al. 1996). Among those factors, different genotypes could be associated to a higher risk of transmission (Renjufi et al. 2001). On the other hand, other investigators have found that although low CD4 and high viral loads were predictive of MTCT, no association with HIV-1 subtype was observed (Tranchat et al. 1999, Guevara et al. 2002, Tapia et al. 2003, Renjufi et al. 2003). Therefore, observations in this issue have not been consistent. Obstetric factors as type of delivery, time of membrane rupture and birth weight, and the use of ACTG 076 protocol during pregnancy and delivery, have been identified as risk factors for MTCT (Fawzi et al. 2001, Zijenah et al. 2004).

The present study assessed the prevalence of subtype C of HIV-1 in the city of Rio Grande, RS, Brazil, through the epidemiological and molecular analysis of HIV-1-positive pregnant women attending the AIDS service of Rio Grande University Hospital (HU/FURG). MTCT patterns were also evaluated according to HIV-1 subtype and other host-related factors of the infected mothers.

MATERIALS AND METHODS

Population, Samples and Variables

This cross-sectional study included HIV-1-positive mothers that attended the AIDS service of HU/FURG from 1998 to June 2004 and their newborn children. Clinical data were obtained from their follow-up medical records. The study was submitted and approved by the research ethic committee of the institution.

The infant HIV infection status was defined as positive, according to guidelines of the Brazilian Ministry of Health, if the individual presented two consecutive tests with detectable viral load or two positive serological tests and a confirmatory indirect immunofluorescence test by 18 months of age. Since 1998 every pregnant woman that attended the institution was tested for HIV-AIDS as recommended by the health authorities.

The independent variables studied were:

a) mother HIV-1 subtype, determined as described below and exposed mothers were defined as those infected with subtype C;

b) use of AIDS Clinical Trial Group (ACTG) 076 protocol;

c) $\log_{10}$ HIV viral load (VL) of the mother in the last three months of pregnancy;

d) CD4 T-cell counts of the mother in the last three months of pregnancy;

e) type of delivery;

f) time of membrane rupture (less than two hours/ two hours or more);

g) children weight at birth.

Use of ACTG 076 was categorized as a) complete: when the mother received zidovudine during pregnancy and at labor, as well the newborn; b) incomplete: when at least one of the three situations where accomplished; c) null: when no zidovudine was taken by the mother and the infant.

Blood Collection and HIV-1 Subtype Determination

Ten milliliters of whole blood were collected from each patient. Fifty microliters were used for CD4 T-cell counts by flow cytometry in a FACSCount apparatus (Becton & Dickinson, Franklin Lakes, NJ) according to the manufacturer's specifications. Five milliliters were used for HIV-1 VL determination by the branched-DNA technique (Quantiplex TM bDNA 340, Bayer, Leverkusen, Germany), also as recommended by the producer. The remaining blood was used for PBMC DNA extraction as described below.

HIV-1 Subtype Determination

Approximately 5 ml of whole blood was used for peripheral blood mononuclear cell (PBMC) extraction. PBMC were isolated by centrifugation in Ficoll-Hypaque (Amersham, Uppsala, Sweden), washed in PBS and then lysed in 200μl of lysis buffer (TE pH 8.1, 0.001% Triton X-100, 0.001% SDS). Fifty micrograms of proteinase K were added to each sample and the proteolysis was carried out at 56°C for 1 h. Proteinase K was inactivated at 95°C for 10 min.

env gene fragments corresponding to the IDR domain of gp41 were amplified in nested PCR reactions using genomic DNA as template. The primers used were JH41F (5'CAG CAG GWA GCA CKA TGG G 3') and JH38R (5'GGT GAR TAT CCC TKC CTA AC 3') (outer), and ENV27F (5'CTG GYA TAG TGC ARC ARC A 3') and MENV19R (5'AAR CCT CCT ACT ATC ATT ATR A 3') (inner). PCR amplifications were conducted in 50μl reaction volumes containing 10X Taq reaction buffer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 12.5 pmol of each respective primer and 1.25U of Taq polymerase. Reactions were carried out under the following conditions: one initial denaturing cycle of 3 min at 95°C, 1 min at 56°C and 1 min at 72°C, followed by 35 cycles of 30 sec at 95°C, 45 sec at 55°C and 1 min at 72°C. A final extension step of 7 min at 72°C was conducted to complete all unfinished PCR products. Inner reactions were carried out under the same conditions, with the exception of the annealing temperature, which was of 56°C. All reactions were carried out in a 9700 DNA thermal cycler (Applied Biosystems, Foster City, CA). Generated PCR products were purified with the Concert Rapid PCR Purification System kit (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations, and analyzed in a 1% agarose gel. DNA products were quantified using the DNA mass ladder (Invitrogen), and 100 ng were used in sequencing reactions with the same primers used in PCR. Reactions were conducted with the BigDye Terminator Cycle Sequencing Reaction kit v.3.0 (Applied Biosystems), and were run in an ABI 3100 Genetic Analyzer apparatus (Applied Biosystems).

Sequences in electronic format were edited in the software SeqMan of the LaserGene package (DNAStar Inc., Madison, WI), and then aligned in ClustalW (Thompson et al. 1994). HIV-1 subtype determination was conducted by aligning query sequences with an HIV-1 subtype reference set obtained at the Los Alamos National Laboratory HIV Database (http://hiv-web.lanl.gov). Phylogenetic analysis using the neighbor-joining method and the Kimura two-parameter correction was conducted with all sequences, and samples were assigned a specific subtype when they grouped with reference sequences from a specific HIV-1 clade. The inference was calculated in the program MEGA 2.0 (Kumar

et at. 2001), and phylogenetic trees were drawn in TreeView (Page 1996). The robustness of the different clades in the tree was assessed by bootstrap with 2,000 replicates under MEGA.

Statistical Analyses

Statistical evaluations were conducted in the package Stata 8.0 (Stata Corp., College Station, TX). Firstly, a descriptive analysis was conducted, where averages and standard deviations were calculated for the numeric variables and the proportions for the categorized variables. Secondly, bivariate analyses were carried out to assess the association between child HIV status and the several studied factors. This was done by calculating the prevalence ratios (PR) and their respective 95% confidence intervals (CI_{95}). In these analyses the chi-square test was used. Finally, a multivariate analysis was conducted between the child HIV status and the diverse associated risk factors adjusted to each other. A backward Poisson regression was used for this. The variables that had a *p*-value lower than 0.05 were kept in the model. The adjusted PR and CI_{95} were then recalculated. The significance of the models was assessed by the Wald test. A 0.05 two-tailed level of significance was applied to all statistical evaluations.

Sequence Data

All *env* gp41 IDR and gp120 sequences were submitted to GenBank and were assigned the accession numbers AY621381 through AY621463.

RESULTS

Characteristics of the Studied Samples

In the studied period a total of 102 children were born from HIV-infected mothers and enrolled. The average age of the mothers was 25.86 years (SD 5.96). Fifty three percent of the children were males. Seventy two percent of the mothers were infected by subtype C viruses. Twelve (11.8%) newborns were HIV-1-positive, slightly over half of the mothers (52.6%) had CD4 T-cell counts between 200 and 499 cells / mm^3 of blood (CDC immunological stage B), and 47.3% had a $\log_{10}$ HIV VL between 4 and 5. The average CD4 T-cell count was 403.8 cells/mm^3 (SD 226) and the $\log_{10}$ HIV VL was 3.2 (SD 1.8). Other characteristics of the studied samples are depicted in Table I.

Risk Factors for MTCT

Prevalence ratio adjusted values can be observed for all studied models in Table II. The $\log_{10}$ HIV VL was a risk factor for mother-to-child transmission. Each log increased the risk of transmission in more than seven hundred percent. Use of ACTG 076 protocol was associated with the HIV status of the child, and a linear trend was detected between the three studied categories. Those with complete protocol had a 99% reduction compared to the null protocol category. All other factors had lost their effect or drastically diminished their PR when adjusted to each other, and thus could not be considered MTCT predictors. CD4 T cell counts, which had shown a p-value = 0.02 in the bivariate analysis, had their PR significantly reduced and their p-values increased when adjusted to other factors.

DISCUSSION

The present study analyzed the effect of several putative risk factors on vertical transmission of HIV-1 in Southern Brazil. We have found that the $\log_{10}$ HIV VL was the best predictor of MTCT, and that each unit increase corresponded to an increase in risk for transmission of 8.5 times. This result is consistent with the findings by several groups (Kamara et al. 2005, Magder et al. 2005, Guevara et al. 2002, Katzenstein et al. 1999, Garcia et al. 1999, Fawzi et al. 2001, Blackard et al. 2001). In contrast to that, Tranchat et al. (1999) found no significant association between maternal HIV VL and vertical transmission. The reasons for the discordant results remain to be elucidated, but they might be associated with the low statistical power of small samples which are characteristic of those studies. For CD4 T-cell counts, we found no significant association. This result is in agreement with that of Katzenstein et al. (1999), whereas other studies have found an

TABLE I

HIV-1 subtype and maternal and infant characteristics of the studied cohort (n=102).

Characteristic	Sample n (%)	Transmitting mothers n (%)	Non-transmitting mothers n (%)
Infant HIV status (n, %)			
Negative	90 (88.2)	—	—
Positive	12 (11.8)	—	—
Time of membrane rupture (n, %)			
$<$ 2 hours	81 (79.4)	10 (12.3)	71 (87.7)
$\geq$ 2 hours	21 (20.6)	2 (9.5)	19 (90.5)
Delivery (n, %)			
Cesarian	39 (38.2)	4 (10.3)	35 (89.7)
Vaginal	63 (61.8)	8 (12.7)	55 (87.3)
Weight at birth (mean, SD)	2940.7 (536.6)	2856.7 (406.9)	2952.8 (553.0)
Mother Infecting HIV-1 Subtype (n, %)			
B	28 (27.5)	2 (7.1)	26 (92.9)
C	74 (72.5)	10 (13.5)	64 (86.5)
Use of ACTG 076 (n, %)			
Null	7 (6.8)	5 (71.4)	2 (28.6)
Incomplete	33 (32.4)	6 (18.2)	27 (81.8)
Complete	62 (60.8)	1 (1.6)	61 (8.4)
CD4 T-cell counts (n, %)			
0-199	18 (19.0)	4 (22.2)	14 (78.8)
200-499	50 (52.6)	3 (6.0)	47 (94.0)
$\geq$ 500	27 (28.4)	0 (0.0)	27.0 (100.0)
CD4 T-cell (mean, SD)	403.8 (226.0)	231.4 (152.5)	417.5 (225.9)
Log_{10} HIV viral load			
0 - 2.99	27 (28.4)	0 (0.0)	27 (100.0)
3 - 3.99	23 (24.2)	0 (0.0)	23 (100.0)
4 - 4.99	35 (36.8)	2 (5.7)	33 (94.3)
$\geq$ 5	10 (10.5)	5 (50.0)	5 (50.0)
Log_{10} HIV viral load (mean, SD)	3.2 (1.8)	5.2 (0.6)	3.1 (1.8)
Infant HIV status (n, %)			
Negative	90 (88.2)	—	—
Positive	12 (11.8)	—	—

association between CD4 counts and MTCT (Renjufi et al. 2001, Tranchat et al. 1999).

Another important finding was the effect of ACTG 076 protocol, which reduced the risk of transmission. A clear dose-response relation was found between categories, and those who had an incomplete treatment had an intermediate risk, when compared with those under no treatment. A conspicuous reduction was observed when mother and child are treated. Magder et al. (2005) have also found a neg-

TABLE II

Bivariate and multivariate-adjusted prevalence ratios (PR) for HIV-1-positive infant status and risk factors.

Risk Factor	Prevalence (n)	Bivariate PR (95% CI)	*p*-value	Multivariate PR (95% CI)	*p*-value
Maternal CD4 T cell		1		1	
counts (cells/mm^3)		0.20 (0.05-0.75)	0.02	0.99 (0.99-1.99)[a]	0.3
Time of membrane rupture					
At delivery	12.9 (10)	1	0.7	1	
Before delivery	9.52 (2)	0.7 (0.16-3.34)		8.89 (0.28-2.84)[b]	0.2
Weight at birth		0.99 (0.99-1.00)	0.6	0.99 (0.99-100)[c]	0.2
Delivery					
Cesarian	11.1 (4)	1		1	
Vaginal	12.9 (8)	1.16 (0.35-3.85)	0.8	0.22 (0.01-3.02)[d]	0.3
Maternal Subtype					
B	7.14 (2)	1		1	
C	14.29 (10)	2 (0.44-9.13)	0.4	2.10(0.24-18.2)[e]	0.5
ACTG 076					
Null	71.4 (5)	1		1	
Incomplete	18.8 (6)	0.26 (0.08-0.86)	0.001	0.02 (0.000-0.47)[f]	0.05*
Complete	1.7 (1)	0.02 (0.002-0.20)		0.008 (0.00-0.22)	
HIV $\log_{10}$ VL (1 $\log_{10}$)		8.53(2.28-31.96)	0.001	8.53 (2.38-31.96)[f]	0.001

[a] Model adjusted to maternal CD4 counts, time of membrane rupture, weight at birth, type of delivery, maternal subtype, ACTG 076, and HIV viral load (n=92); [b] Model A minus maternal CD4 counts (n=92); [c] Model B minus time of membrane rupture (n=92); [d] Model C minus weight at birth (n=95); [e] Model D minus type of delivery (n=95); [f] Final Model, PACTG 76 and HIV viral load (n=95); *p-value for linear trend.

ative association between antiretroviral therapy and vertical transmission.

In regard to the prevalence of subtype C, our group has reported that this subtype represented 22% of the samples in 1997 (Martínez et al. 2002). In this study, seven years later, a subtype C prevalence was found over 70%. Therefore, the increase of subtype C is evident in this part of Brazil. Similar subtype C dissemination has been reported in Southern and Eastern Africa, regions where the HIV/AIDS epidemic affects a large portion of the population (Peeters 2001, Perrin et al. 2003).

There was no association in our study between infecting HIV-1 subtype and increased risk for MTCT. This is in agreement with data by Tranchat et al. (1999), Murray et al. (2000) and Tapia et al. (2003). Other studies, however, did find a relationship between subtype and MTCT, mostly in Africa where a variety of subtypes exists (Blackard et al. 2000, Yang et al. 2004, Renjufi et al. 2004). It is important to note, however, that the HIV-1 subtypes compared in those studies did not include subtype B, and thus further differences in MTCT might still be of importance.

Obstetric factors are also considered a risk factor of MTCT. Some studies have found no association (Renjufi et al. 2004, Yang et al. 2004), while others have (Zijenah et al. 2004). Fawzi et al. (2001) detected an association between prematurity and transmission, but did not find it for time of membrane rupture.

In this study, neither the type of delivery, time

of membrane rupture, nor birth weight were associated with HIV transmission. However, only two newborns were positive in the $\geq$ 2h membrane rupture group. Therefore, the possibility of lack of power must be considered. Another explanation for the absence of association between vertical transmission and obstetric factors in this study may be the described association between HIV subtype C and *in-utero* transmission (Renjufi et al. 2004). As it has been reported in this study most of the positive cases were with subtype C, masking the effect of time of membrane rupture and type of delivery. Under such scenario, specific and fast-acting MTCT prevention programs should take place in southern Brazil, where the prevalence of subtype C is high (Soares et al. 2003, Martínez et al. 2002).

In summary, this study has shown that the most important factors involved in MTCT are maternal HIV viral load and the use of ACTG 076 protocol. Although the expansion of HIV-1 subtype C in southern Brazil highlights the importance of monitoring the dynamics of the HIV/AIDS epidemic in that part of the country, the infection with this subtype does not seem to have an increased risk of MTCT.

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RESUMO

Diferentes subtipos do virus da imunodeficiência humana do tipo 1 (HIV-1) podem ter propriedades biológicas, imunológicas e patogênicas distintas. A eficiência da transmissão materno-infantil (TMI) pode estar entre estas propriedades, porém resultados escassos e controversos foram descritos até o momento. Neste estudo, 102 crianças nascidas de mães infectadas pelo HIV-1 entre 1998 e 2004 na cidade do Rio Grande, Brasil, foram analisadas para fatores de risco potenciais associados à TMI. Aquela região geográfica é caracterizada por uma alta proporção de indivíduos infectados pelo subtipo C do HIV-1, permitindo a comparação entre os subtipos B e C e sua influência na transmissão vertical do vírus. A análise também incluiu parâmetros clínicos, obstétricos e imunológicos. Análises de regressão multivariada foram conduzidas para avaliar a influência daqueles parâmetros na TMI, e as razões de prevalência (RP) e intervalos de confiança de 95% (IC95) foram também calculados. Um prevalência surpreendentemente alta do subtipo C acima dos 70% foi encontrada. Somente a carga viral do HIV e o uso de protocolo ACGT 076 maternos forma preditivos de TMI. O subtipo do HIV-1 e a contagem de células T CD4+ não foram associados a um risco aumentado de transmissão. Embora uma clara expansão do subtipo C seja evidente no sul do Brasil, esta não parece estar correlacionada com risco aumentado de transmissão vertical.

Palavras-chave: HIV, transmissão materno-infantil, transmissão vertical, subtipo C, epidemiologia molecular.

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