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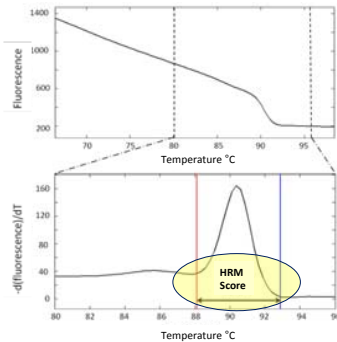
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## BACKGROUND

In resource-limited settings, many HIV-infected children die before two years of age. In this study, we analyzed the association between HIV diversity and survival in HIV-infected infants. HIV diversity is usually evaluated by sequencing individual viral variants. We developed a rapid high resolution melting (HRM) assay that can be used to assess HIV diversity without sequencing. In the HRM assay, regions of the HIV genome are amplified in the presence of a fluorescent dye. The resulting HIV amplicons are then warmed over a range of temperatures, and an HRM score is determined by analysis of the resulting melting curve (Figure 1).

Figure 1. HRM method



The HRM assay is highly reproducible, and HRM scores are significantly associated with sequence-based measures of HIV diversity [1]. In this study, we used the HRM assay to measure the level of diversity in three regions of the HIV genome, and examined the relationship between HIV diversity and infant survival.

## METHODS

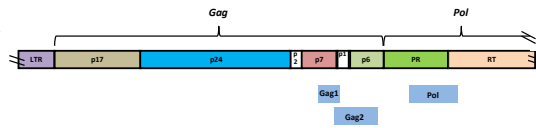
### Study samples

Samples were obtained from 31 Ugandan infants in the HIVNET 012 clinical trial who were HIV-infected by 6-8 weeks of age. Most of these infants received single dose nevirapine (sdNVP) for prevention of HIV mother-to-child transmission [2]. The plasma samples tested in this study were collected at 6-8 weeks, 12 months, and 18 months of age.

### HRM analysis

The HRM assay was performed using a LightScanner instrument (Idaho Technology, Salt Lake City, UT) as previously described [1]. The HRM assay was used to analyze diversity in two regions of HIV *gag* (Gag1, Gag2) and a region in HIV *pol* (Pol, Figure 2).

Figure 2. Regions analyzed



## RESULTS

### Analysis of HIV diversity in infants

HRM scores for samples from 6-8 week-old infants were higher than those obtained using plasmid controls. Twenty-one of the 31 infants were alive at 12 months and had samples available for testing from 12 or 18 months of age. Higher HRM scores were associated with older age (beta=0.37, P=0.005; for Gag2: beta=0.47, P=0.006; for Pol: beta=0.24, P=0.016; where beta is the estimated mean increase in the HRM score associated with one year increase of age).

Table 1. HRM assay results\*

Region analyzed	N	Gag1	Gag2	Pol
Corresponding region in HXB2	1998 - 2096	2071 - 2278	2373 - 2597	
HRM amplicon size	99 bp	208 bp	225 bp	
HRM scores for plasmids	4	3.8 (3.8, 3.8)	3.4 (3.2, 3.8)	3.5 (3.4, 3.5)
HRM scores for 6-8 week old infants	31	4.7 (4.4, 7.3)	4.2 (3.6, 6.3)	4.1 (3.3, 6.7)
HRM scores for 12 month old infants	17	5.2 (4.3, 7.1)	4.6 (3.8, 6.4)	4.3 (3.3, 5.9)
HRM scores for 18 month old infants	15	5.1 (4.6, 6.3)	4.9 (4.2, 7.8)	4.4 (4.0, 5.9)

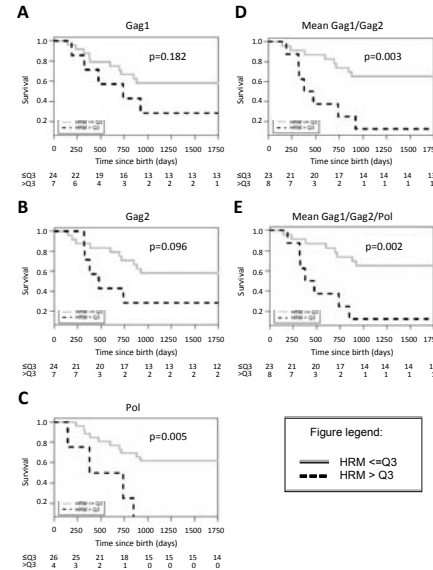
\*Median and range are shown for HRM scores

We found no significant associations between HRM scores (above vs. below the third quartile) and the following variables: infant CD4 cell count % at birth, infant HIV viral load at 14 weeks, sdNVP exposure, maternal CD4 cell count at delivery, maternal HIV viral load at delivery, HIV infection *in utero*, infant NVP resistance at 6-8 weeks of age, and HIV subtype (data not shown).

### Analysis of HIV diversity and infant survival

We analyzed the association between HRM scores obtained at 6-8 weeks of age and infant survival. In the Kaplan-Meier analysis shown below, infants with HRM scores above the 75<sup>th</sup> percentile (above the third quartile, >Q3) were characterized as having high HRM scores, and infants below that cutoff were characterized as having low HRM scores. Higher HRM scores were significantly associated with reduced infant survival for the Pol region, the mean of the Gag1 and Gag2 regions, and the mean of all three regions (Figure 3).

Figure 3. Kaplan-Meier analysis of HRM scores and infant survival\*



\*The X axis shows the time since birth in days (infant age); the Y axis shows the survival probability. The number of infants still alive in each group ( $\leq$  Q3, >Q3) at each time point is shown below each graph.

In a multivariate hazard model that included HIV viral load at 14 weeks of age and HRM score, higher HRM scores (for Gag2, mean of Gag1 and Gag2, and the mean of Gag1, Gag2, and Pol) were associated with death (Table 2).

Table 2. Logistic regression analysis of HRM scores and infant survival

HRM score at 6-8 weeks	N	Hazard Ratio (95% CI)		P	N	Hazard Ratio (95% CI)		P
		Unadjusted	Adjusted <sup>1</sup>			Unadjusted	Adjusted <sup>1</sup>	
Gag1	31	2.0 (0.7, 6.0)	0.19	26	2.1 (0.7, 6.6) <sup>2</sup>	0.12	2.6 (0.8, 9.2) <sup>3</sup>	0.12
Gag2	31	2.5 (0.8, 7.3)	0.11	26	3.5 (1.1, 10.9) <sup>2</sup>	0.03	2.4 (0.7, 7.8) <sup>3</sup>	0.15
Pol	30	4.7 (1.4, 15.8)	0.01	25	3.4 (1.0, 11.9) <sup>2</sup>	0.06	2.7 (0.7, 10.6) <sup>3</sup>	0.15
Mean (Gag1, Gag2)	31	4.2 (1.5, 11.9)	0.006	26	8.7 (2.6, 28.6) <sup>2</sup>	0.0004	3.4 (1.1, 10.8) <sup>3</sup>	0.04
Mean (Gag1, Gag2, Pol)	30	4.6 (1.6, 13.2)	0.004	26	6.9 (2.1, 22.9) <sup>2</sup>	0.002	1.7 (0.5, 5.3) <sup>3</sup>	0.40

<sup>1</sup>Multivariate models: included HRM score measured at 6-8 weeks of age (binary,  $\leq$  75<sup>th</sup> percentile vs. >75<sup>th</sup> percentile) and HIV viral load measured at 14 weeks of age (log<sub>10</sub> scale). N: number of infants included in the model

<sup>2</sup>Hazard ratio, 95% CI, and P value for HRM score at 6-8 weeks of age.

<sup>3</sup>Hazard ratio, 95% CI, and P value for HIV viral load at 14 weeks of age (log<sub>10</sub> scale).

## CONCLUSIONS

HIV diversity in the *gag* and *pol* regions was low in 6-8 week old infants and increased with age. Higher levels of HIV diversity in 6-8 week old infants were associated with reduced infant survival. Further studies are needed to assess the relationship between HIV diversity in other regions of the HIV genome and survival in HIV-infected infants.

## REFERENCES

- Towler WI, et al. Analysis of HIV diversity using a high resolution melting assay. AIDS Res Human Retroviruses 2010; 26:913-918.
- Guay LA, et al. Intrapartum and neonatal single-dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in Kampala, Uganda: HIVNET 012 randomised trial. Lancet 1999; 354:795-802.