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Short Communication

Early Screening of HIV-1 from Dried Blood Spots in Infants Born to HIV-1 Positive Mothers from North Indian States

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Abstract

Early diagnosis of HIV-1 in infants born to HIV-1 positive mothers is a challenging job in the resource limited settings in the absence of signs and symptoms of the disease at the time of birth. Serological tests in children younger than 18 months are not decisive and reliable due to maternally transferred antibodies. This study reports DNA PCR on Dried Blood Spots (DBS) obtained from infants born to HIV-1 infected mothers from some of the north Indian states. Of the 1646 DBS tested, 269 (16.34%) were found to be HIV-1 positive and the positivity was slightly higher in males (17.4%) than females (14.8). Based on feeding practices the study revealed a statistically significant higher transmission rate in infants on mixed feed compared to breast or replacement feed.

ABBREVIATIONS

HIV: Human Immunodeficiency Syndrome Virus; DBS: Dried Blood Spots; MTCT: Mother To Child Transmission; ART: Ante Retroviral Therapy; ELISA: Enzyme Linked Immuno Sorbent Assay; EID: Early Infant Diagnosis; ICTC: Integrated Counseling and Testing Centre; PPTCT: Prevention of Parent to Child Transmission; WBS: Whole Blood Samples

INTRODUCTION

Women and children constitute the most affected patient population among all the HIV-1 (Family: Retroviridae, Genus: Lentivirus, Species: Human Immunodeficiency Virus 1) seropositive individuals all over the world [1]. HIV-1 infected mothers may transmit the virus to their children during prepartum (pregnancy), intrapartum (childbirth) or postpartum (breastfeeding) [2-5]. The risk of HIV-1 transmission from mother to the child, without therapeutic intervention is high that may range between 15% and 25% in developed countries and even higher from 25% to 45% in developing countries [3,5]. Of all the HIV-1 infected patients, neonates/infants are the most vulnerable as the virus replicates at a higher rate in the first year of their life compared to the adults, contributing to a higher viral load and rapid disease progression [2,6,7]. In parts of the world where diagnosis, care and treatment are not available, 35% of infected children die in the first year of life, 50% by the age of two and 60% by the age of three years [8,9].

Infants who acquire HIV-1 infection *in-utero* rapidly progress towards the disease during the first year of their lives [2,7,10]. CHER trial has shown that early ART provided to HIV-1 infected infants at an early stage results in sharp reduction in mortality and morbidity [11]. If the HIV-1 infection status of an infant is known at an early stage, early ART curtails the disease progression and the child receives the opportunity to live a healthy life. However, the early diagnosis in these infants remains difficult, as the most infected babies appear healthy and exhibit no sign and symptom of the disease at birth [3]. Appropriate HIV-1 diagnostic methods for infants differ from those that are used for children, adolescents or adults because in infants the trans-placental transmission of maternal antibodies or antibodies transmitted postnatal through breastfeeding may be detectable in the infant's bloodstream until the age of 18 months. In these infants, the results of conventional methods of HIV-1 diagnosis based on serology (ELISA) may be reactive due to the passively acquired maternal antibodies. For these reasons, the methods of HIV-1 diagnosis based on serology are not reliable in infants and search for a substitute becomes essential [4]. The sensitivity of quantitative RNA assays may vary

from 90% to 100%, which are carried out after the 2-3 months of age. Assays based on RNA detection are reported to be affected by ART treatment in mothers [4,12,13]. In addition, these methods of diagnosis require large amount of blood, which is difficult to draw in infants.

Monitoring and diagnosis of vertically HIV-1 infected children during the first year of life is best performed by DNA PCR. It is the most sensitive technique which detects HIV-1 proviral DNA integrated into the human genome. Specificity of this test is reported to be 99.8% at birth and 100% at 1, 3, and 6 months. Sensitivity of the test performed at birth is 55% but increases to more than 90% by 2 to 4 weeks of age, and 100% at 3 and 6 months of age [12-14]. Since sample withdrawing is difficult in infants, the spotting of whole blood through heel prick onto filter paper i.e. DBS is used which is economical and advantageous over venipuncture [15-17]. PCR performed on DBS is comparable to PCR performed on WBS and shown to be 100% sensitive and 99.6% specific. DBS is easy to prepare, store and ship via courier or mail to the testing laboratories in a resource limited setting without refrigeration and fear of spread of infection [8,15,17]. In the current study, DBS of infants and children (aged 6 weeks to 18 months), born to HIV-1 seropositive mothers, were tested for early diagnosis of HIV-1 by DNA PCR. The results have been analyzed and presented based on state-wise prevalence of HIV-1in infants in northern part of India. In addition, gender and feeding practices of infants adopted by various socio-economic strata are also taken into consideration. Of all the feeding practices such as breast feeding, mixed feeding and replacement feeding, mixed feeding practice was found to be worse as the HIV transmission rate was highest in this group of patients in this study.

MATERIALS AND METHODS

DBS from infants and children aged 6 weeks to 18 months, born to HIV-1 seropositive mothers were received at National Centre for Disease Control (NCDC) from various ICTC/PPTCT centers of northern states of India (Delhi, Punjab, Chandigarh, Rajasthan, Uttar Pradesh and Uttrakhand). The study was carried out at NCDC with proper ethical clearance for early diagnosis of HIV-1 on the samples collected from January, 2011 to July 2012. According to National AIDS Control Organization guidelines for EID, all the infected mothers received a single dose of Nevirapine during the labor to curtail the vertical transmission of HIV-1. All the exposed infants also received Nevirapine within 72 hours of birth followed by Cotrimoxazole as prophylaxis [3].

Blood samples were taken by the heel prick of the children and spotted onto the filter paper. This was followed by drying the spots on filter paper. The DBS were tested qualitatively using the kit Amplicor HIV-1 DNA Test, version 1.5 (Roche Molecular Systems, USA) as per the manufacturer's protocol. Testing procedure consisted of four major processes – sample preparation, amplification of the target DNA using HIV-1 specific primers, hybridization of the amplified product to the oligonucleotide probes specific to the target and detection of the probe bound amplified products.

HIV-1 DNA was isolated by washing the DBS using BLD wash solution (Sodium Phosphate solution, <0.4% Detergent, 0.05% Sodium Azide) provided in the kit to extract the leucocytes which were then mixed with the Extraction Reagent (Tris-KCl buffer, 0.1% detergent, 0.01% Proteinase K and 0.1% ProClin 300) and incubated for 30 minutes at 60°C in a dry heat block to extract the DNA. DNA sample was added to the amplification mixture containing the primers, dNTPs, buffer and DNA polymerase (rTth). The biotinylated primers used for DNA amplification were SK145 (5`-AGTGGGGGGACATCAAGCAGCCATGCAAAT-3`) and SKCC1B (5`-TACTAGTAGTTCCTGCTATGTCACTTCC-3`), which amplified a sequence of 155 nucleotides within the highly conserved region of *gag* gene. The samples were prepared and amplified using the Gold Plated 96 well Gene Amp PCR System 9700 thermal cycler under the following cycling conditions:

Hold	3 min. at 50°C
5 cycles	10 sec at 94°C, 10 sec at 50°C, 10 sec at 72°C
35 cycles	10 sec at 90°C, 10 sec at 54°C, 10 sec at 72°C
Hold	15 min at 72°C

Following the PCR amplification, the amplicons were chemically denatured by the addition of Denaturation Solution (1.6% (w/w) Sodium Hydroxide EDTA Thymol blue) incubating for 10 minutes at room temperature to form single-stranded DNA. 100 µL of Hybridization buffer (Sodium phosphate solution, 0.2% Solubilise, 25% Sodium Thiocyanate) was added to each well of microwell plate (MWP). Then 25µL of denatured amplicon were added to each well of MWP coated with HIV-1 specific, SK102, oligonucleotide probe (5`-GAGACCATCAATGAGGAAGCTGCAGAATGGGAT-3`). The MWP was gently tapped for about 10-15 times until the color changes from blue to light yellow. The MWP was then incubated for about 1 hour at 37°C. Following the hybridization reaction, the MWP was washed 5 times manually by adding 250-300 µL of working wash solution (2% Phosphate Buffer, 9% Sodium Chloride, EDTA, 2% Detergent, 0.5% ProClin 300). This was followed by addition of 100 µL of Avidin-Horseradish Peroxidase (HRP) Conjugate (Tris-HCl buffer, 0.001% AV-HRP conjugate, Bovine gamma globulin, Emulsit 25, 0.1% Phenol, 1% ProClin 150) and incubating for 15 minutes at 37°C. Avidin-HRP conjugate binds to the biotin-labeled amplicon hybridized to the target-specific oligonucleotide probe. The MWP was washed again to remove unbound conjugate and 100 µL of substrate solution {containing Citrate solution, 0.01% hydrogen peroxide, 0.1% ProClin 150 and 0.1% of 3,3',5,5'-tetramethylbenzidine (TMB) and 40% w/w Dimethylformamide} was added to each well. It was incubated in dark at room temperature for about 10 minutes to develop color. The reaction was stopped by addition of 100 µL of a stop solution (4.9% Sulphuric acid) and subsequently the absorbance at 450nm was measured within 30 minutes at 450nm in an automated microwell plate reader. The samples showing the Optical Density (OD) value of <0.2 were negative while the samples showing OD values of ≥0.8 were positive for HIV-1 DNA as per directions described in the kit. However, the threshold OD values of ≥0.8 were adjusted by routine molecular laboratory testing methods to ensure reporting of correct positive results [17].

RESULTS

A total of 1661 DBS samples were received, out of which, 1646 were tested and 15 were excluded due to poor sample quality, quantity or inconclusive results. Out of the 1646 DBS tested, 269

(16.34%) were found to be positive for HIV-1. The distribution of HIV-1 positivity varied in several States. Uttrakhand and Rajasthan States showed the higher positivity rate of 33.33% and 21.97% respectively. Samples from Uttarakhand state presented the lowest positivity rate compared to the other Northern States included in this study. In Delhi, UP, Punjab and Chandigarh, the positivity rate ranged from 13% to 16% (Figure 1).

Male infants and children were found to show higher positivity rate, approximately 17.4% compared to the female infants, about 14.8% but the difference was not statistically significant (p = 0.1504). Considering the feeding practice, infants and children on mixed feed (27/51; 34.6%) had an about 2 fold higher frequency of HIV-1 detection in comparison to exclusive breast feeding (92/431; 17.5%) or replacement feeding (133/761; 14.7%), respectively (Table 1). The frequency of HIV-1 detection was statistically significant between replacement feed and mixed feed (p <0.0001), and between breast feed and mixed feed (p <0.001) but it was not statistically significant between breast feed and replacement feed (p <0.1774). Taken together, HIV transmission rate in infants and children was highest among mixed fed followed by breast fed and least among replacement fed.

DISCUSSION

In developing countries having resource-limited settings, EID is a challenging and critical job in order to start an effective therapy. The lack of early diagnosis in infants born to HIV-infected mothers puts HIV-infected infants at higher risk of mortality, which is estimated to reach to 35.2% before reaching the age of 1 year [18]. Under such circumstances, priority and emphasis is on EID so as to administer life saving drugs against HIV to reduce early mortality and improve overall health of HIV-infected infants and children. In the current study, HIV-1 positivity in infant and children in two northern states namely Rajasthan and Uttrakhand was found comparatively to be far higher 21.97% and 33.33% respectively than four other states namely Delhi, Punjab, UP and Chandigarh, which had almost 13-16% HIV positivity. The higher mother to child HIV transmission in Rajasthan to some extent may be attributed to higher rate of breast feeding habits by mothers compared to other states where replacement feed was more frequent in HIV-1 positive mothers. The HIV positivity from Uttrakhand though seems to be even higher, however, the overall sample size from this state was very low (Figure 1), which may not appropriately represent vertical HIV transmission in this state, thus necessitating a bigger sample size in future studies that may provide true representation. The other risk factors, which have been attributed to increased MTCT include higher levels of maternal viraemia, low maternal CD4 count, co-existing other sexually transmitted disease, invasive intrapartum procedures and rupture of membrane [5,7,19] however, no such data could be made available in the current study. Additionally, the data on caesarian vs. normal delivery was not available; nonetheless, the issue is complex and remains unclear. It is worth noting in the present study that no statistical significant difference was observed in the HIV positivity between males and female contrasting various other reports that show significant higher proportion of HIV positivity in males than in females [20-22].

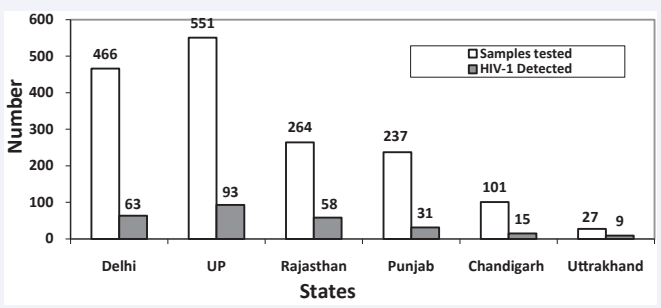

Figure 1 Detection of HIV-1 in DBS of infants of HIV-1 infected mothers from various North Indian states.

Table 1: Consolidated DBS test data based on gender and feeding practice.

Patient Information	HIV-1 (+)	HIV-1 (-)	*p* values
No. of samples (n=1646)	269 (16.3%)	1377	
Gender*			
Male	167 (17.4%)	788	Male *vs* Female p =0.1504
Female	102 (14.8%)	586	
Feeding practice**			
Breast feed (BF)	92 (17.5%)	431	BF *vs* RF (p =0.1774)
Replacement feed (RF)	133 (14.8%)	761	
Mixed feed (MF)	27 (34.6%)	51	MF *vs* BF (p <0.001) MF *vs* RF (p <0.0001)

* based on available gender information;
** based on available feeding practice
% of HIV positivity is shown in parentheses
p values were calculated by χ^2 calculations (Two-way contingency table)

Another important issue in the current study is related with feeding practice by HIV positive mothers to their infants and children. In the present study, statistically significant higher rate of transmission was observed in infants on mixed feed compared to both breast feed and replacement feed. Higher HIV transmission rate has been reported in several other studies too [19,23]. Mixed feeding practice which includes both breast feeding as well as replacement feeding seems to pose increased risk to infants of acquiring HIV infection. Since hygiene is a critical issue during replacement feeding, if not practiced hygienically, the gut may get inflamed by some bacterial infection. On time of alternate breast feeding, an inflamed gut may become easier target of HIV transmission from mother's milk [23-25]. Although replacement feed is recommended to be the most ideal alternative to curtail the transmission of HIV-1 but in developing countries including India, it is not recommended due to lack of good hygienic practices [3,5,26]. Contrary to recommendation, however, in the present study the replacement feeding was found to be better and safer in terms of less HIV transmission compared to breast feed or mixed feed. In resource limited settings exclusive breastfeeding is significant as it provides all the essential nutrients and immunity to the child but at the same time the practice puts the children to higher risk of acquiring HIV infection [3,26]. Replacement feeding practice is a socio-economic matter, therefore, in resource-limited settings it may

not always be possible at every level and breast feeding becomes the only choice. In such cases mothers may be recommended for exclusive breast feeding but they should also be counseled to know the importance of periodic monitoring, because HIV-1 exposed infants, who have tested negative may test positive in next screening, if breastfeeding practice is continued.

CONCLUSION

This study shows that significant number of young infants and children are prone to acquiring HIV-1 infection from their mothers. Most importantly postpartum transmission is critical because it can be avoided by judicious decision making not only by clinicians but also by proper counseling of the mothers. ART given to the infants in early period of their life may prove very useful for their healthy survival and increased life expectancy if diagnosed early. Therefore, early diagnosis is critical for early treatment, in reducing the number of deaths and lessening the economic burden as well. Knowing the infectivity status of infants also helps the parents to provide care and attention to their infant.

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