Results and Discussion
4. RESULTS AND DISCUSSION

It is more than 30 years since the first cases of AIDS were reported in 1981 in the USA; HIV-1 became the new plague and killed more than 25 million people throughout the world. Today it is possible to suppress the HIV infection under the detection level; however, patients have to be treated lifelong and it is still not possible to cure the infection. HIV can be transmitted from a HIV positive mother to her infant either during pregnancy or during labour and delivery, or by breastfeeding. Mother to child transmission is the most common route of HIV infection in infants. Detection of HIV-1 specific nucleic acid by PCR is the method of choice for the diagnosis of HIV-1 infection in infants. PCR is a highly sensitive method for the detection of HIV-1 in blood. However, blood separation techniques require extensive laboratory support systems and are difficult when a limited volume of blood is available, which is often the case for infants. The use of blood samples on filter paper has many advantages for the detection of HIV-1 infection.

The guidelines of WHO recommended routine early diagnosis and treatment of HIV positive infants less than 1 year of age (www.who.int). Nucleic acid based testing is the gold standard for early diagnosis and commercially available assays (bDNA, Roche Amplicor and Cobas) are relatively expensive
which require significant infrastructure and technical expertise to allow transfer of technology to resource-limited settings.

The main objective of the study is to know whether whole blood spotted on filter papers can be effectively used for the identification of HIV-1 infection in infants. In whole blood method, entire 200µl is used for DNA extraction whereas in filter papers only a fraction of 10 µl is used. The purpose of the analysis is also to know whether individual gene fragment of HIV is comparable to whole blood.

The sensitivity of the assay was determined by HIV exposed children with known HIV-1 infection and the specificity of the assay was determined by healthy HIV-1 negative children born to HIV positive mothers.

The second objective is to evaluate the liver function profiles to assess the extent of abnormality which may arise due to the treatment of single dose nevirapine received by mother and infant pairs. The results were compared with the normal control mothers and their infants.

As to the third objective, the hematological and immunological status were tested and the correlation between total lymphocyte count and CD4 cell count were found out.

As to the fourth objective, quantitative HIV RNA PCR was performed to measure the viral load for HIV-1 positive infants and their mothers and the correlation between the viral load and CD4 cell count was identified. The drug
resistant mutations of HIV positive mothers and their HIV positive infants were also identified and this was used for their future antiretroviral treatment.

4.1 Demographic characteristics of Mothers

The comparison of age, occupation, breast feeding practices between HIV positive and HIV negative mothers were carried out. The results are given in Table 4.1. The mean age for HIV positive mothers (n=125) was 25.2±3.6 and the age range was between 19 and 34 years. For HIV negative mothers (n=50) it was 24.8±2.9 and the age range was between 19 and 30 years. There was no significant difference between HIV positive and negative mothers in terms of age.

With regard to occupation among the HIV positive mothers, 34.4% (n=43) were coolies and 65.6% (n=82) were home makers. In the case of HIV negative mothers, 32% (n=16) were coolies and 68% (n=34) were home makers. Both the groups were from the same economic background and there was no significant difference in the occupation between HIV positive and negative mothers.

The occupation of the spouse of HIV infected mothers was found to be as follows:

Out of 125 HIV infected males (father of HIV exposed infants), 101 were coolies, 15 were truck drivers and 9 had passed away.

Among 50 HIV negative males (father of HIV non exposed infants), 40 were coolies and 10 were truck drivers.
There was no significant difference in literacy between HIV positive and negative mothers ($p > 0.05$) in this study. Among 125 of HIV positive mothers, 25 had primary education (up to $5^{th}$ standard), 35 had studied up to $8^{th}$ standard and 48 had completed high school education and 17 were illiterate. In HIV negative mothers, 15 had primary education, 25 had studied up to $8^{th}$ standard and 4 had finished high school education and 6 were illiterate.

4.2 Breast feeding practices of Mothers

The breast feeding practices were found to be lesser in HIV positive mothers than in HIV negative mothers. Among 125 HIV positive mothers, 70 had breastfed their infants and 55 had not breastfed their infants whereas in HIV negative mothers 45 had breastfed and 5 had not breastfed their infants. Significant difference was observed in the present study ($p<0.001$), the breast feeding practices were higher in HIV negative mothers than in HIV positive mothers.

Out of 70 breastfed infants, 30 of them were practiced with exclusive breast feeding till 5 months, 40 mothers were given mixed feeding and weaned their infants by 4-6 months whereas in HIV negative mothers (n=45), 33 of them were practiced exclusive breastfeeding till 6 months, 12 mothers were given mixed feeding and weaned their infants by 8-10 months.
Table 4.1 Demographic characteristics of HIV positive mothers and controls

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HIV positive mothers</th>
<th>Controls</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>25.2±3.6</td>
<td>24.8 ± 2.9</td>
<td>0.476^</td>
</tr>
<tr>
<td>Range</td>
<td>19-34</td>
<td>19-30</td>
<td></td>
</tr>
<tr>
<td><strong>Occupation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coolie</td>
<td>43 (34.4%)</td>
<td>16 (32%)</td>
<td>0.087^</td>
</tr>
<tr>
<td>Home maker</td>
<td>82 (65.6%)</td>
<td>34 (68%)</td>
<td></td>
</tr>
<tr>
<td><strong>Literacy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Literate</td>
<td>108 (86.4%)</td>
<td>44 (88%)</td>
<td>0.077^</td>
</tr>
<tr>
<td>Illiterate</td>
<td>17 (13.6%)</td>
<td>6 (12%)</td>
<td></td>
</tr>
<tr>
<td><strong>Breast feeding practices</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast fed</td>
<td>70 (56%)</td>
<td>45 (90%)</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Not breastfed</td>
<td>55 (44%)</td>
<td>05 (10%)</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as Mean ±SD.

*** P < 0.001– Statistically Significant compared with control group
^ Non significant compared with control group

4.3 Effect of nevirapine administration

Skin rashes were observed in 8 (6.4 %) HIV positive mothers and it was developed less than seven weeks after administration of a single dose nevirapine. The other side effects of nevirapine such as fever, diarhoeah, loss of appetite and stomach pain were found in 6/125 (4.8%) of HIV positive mothers. Three studies have reported that nevirapine-associated skin rashes were more common in women (Bersoff et al., 2001; Mazhude et al., 2002; Knudtson et al., 2003). Women with CD4 counts of >250 cells/mm³ had 9.8 times higher skin rashes than women with lower CD4-cell counts (Stern et al., 2003). Bersoff et al. (2001) found out those higher CD4-cell counts were associated with increased risk of severe skin
rashes. Weerawat et al. (2007) found out that 37.7% patients had developed skin rashes and of these 6.6% of them had developed nevirapine-associated Steven-Johnson syndrome in his study.

The percentage of the skin rashes of the present research was found to be lesser when compared with Weerawat et al. (2007) study. The association of skin rashes and higher CD4 counts of the present findings are supported by Stern et al. (2003) study.

4.4 Determination of HIV-1 infection in infants by nested PCR

The results of PCR for the present study are interpreted as follows:

- If any two regions of HIV - pol, gag, and env genes are amplified, the samples are identified as HIV-1 positive.
- If none of the three viral regions is amplified, the samples are considered as HIV-1 negative.
- The positive samples are repeated again with another sample for confirmation.
- Dual concordant results by PCR are classified as HIV infection.

Blood was collected in EDTA tubes from HIV exposed infants and controls, which was spotted onto S&S Isocode stix, protein saver card 903 and FTA card filter papers. DNA was extracted from whole blood and the blood
spotted three filter papers. Nested PCR was performed and the results obtained from the blood spotted filter papers were compared with conventional whole blood method.

4.4.1 Results obtained with whole blood using \textit{gag}, \textit{env} and \textit{pol} genes

DNA was extracted from the whole blood of HIV exposed infants (n=125) and the control children (n=47) and nested PCR was performed with \textit{gag}, \textit{env} and \textit{pol} genes. Out of 125 HIV exposed infants, 13 (10.4\%) were found to be HIV-1 positive and 112 (89.6\%) were HIV-1 negative in all three genes. The age of HIV-1 positive infants ranged from 15 days to 12 months and 7/13 were male 6/13 were female infants.

The age at which the infants were identified with the HIV-1 infection was as follows:

One was found to be positive at 15 days, 2 were at 1 month, 1 was at 42 days, 1 was at 2 months, 1 was at 3 months, 2 were at 6 months, 2 were at 7 months, 2 were at 8 months and 1 was at 12 months.

Fischer \textit{et al.} (2004) suggested that the amplification of the integrated viral genome by PCR is the preferred method for early diagnosis of HIV infection in infants. Nielsen \textit{et al.} (2000) concluded that PCR techniques using whole blood have been the gold standard method to detect HIV infection in infants born to infected mothers. Ceffa \textit{et al.} (2012) also found out that the whole blood is the gold standard method for HIV infant diagnosis. In the present study, initially the
whole blood was used to identify the infection of HIV-1 in exposed infants and control children.

4.4.2 Results obtained from whole blood spotted on filter papers using gag gene

The collection of small amounts of whole blood on filter paper is inexpensive and offers a logistically simple approach for the collection, storage and shipment of large numbers of neonatal samples. The sensitivity, the specificity, NPV and PPV of the three filter papers of the present research are presented in Table 4.2.

DNA was extracted from the whole blood spotted S&S isocode stix filter paper and nested PCR was performed for HIV exposed infants (n=125) and the control children (n=47). Out of 13 HIV-1 positive infants 11 (8.8%) were found to be HIV-1 positive and 114 (91.2%) were HIV-1 negative. The sensitivity was 86.66% and the specificity was 100 %. The two HIV-1 positive infants’ samples could not be amplified in gag gene and it had false negative results. The ages of these two male infants were 2 weeks and one month respectively.

Two punches were used for DNA extraction from whole blood spotted protein saver card 903 and nested PCR was performed for HIV exposed infants and the control children and the results were concordant with the whole blood. Protein saver card showed 100 % of sensitivity, specificity, negative predictive value and positive predictive value. Two punches were used for DNA extraction from whole blood coated FTA card and nested PCR was performed for HIV
exposed infants and the control children; it showed 100 % of sensitivity, specificity, negative predictive value and positive predictive value.

The results of HIV-1 DNAPCR of whole blood spotted on S&S isocode stix filter paper showed 86.6% of sensitivity and 100% of specificity but using protein saver card 903 and FTA card showed 100% in both sensitivity and specificity, compared to the results obtained from whole blood samples. The discordant results of two infants’ samples obtained from S &S isocode paper were retested second time after a month and similar results were observed. The samples did not have the inhibitors of PCR as the β-globin gene was amplified from these samples. Mini et al. (2008) reported that the sensitivity and the specificity of whole blood coated on S & S isocode stix filter paper were 95 and 100 % respectively. In another study the sensitivity and the specificity of the isocode were 90 and > 98%, respectively (Mehta et al. 2009). Qizhang et al. (2008) observed that the sensitivity of the protein saver card 903 was 75 % and the specificity was 100 %.

The findings of the present research are supported by the work of Anitha et al. (2011) who performed the test using HIV-1 DNA PCR of whole blood spotted on protein saver card and reported 100 % of sensitivity and specificity.
Table 4.2 Results of whole blood spotted on filter papers for the detection of *gag* gene in HIV-1 DNA by PCR.

<table>
<thead>
<tr>
<th>Specimen Source</th>
<th>Total no. of samples</th>
<th>HIV-1 Positive</th>
<th>HIV-1 Negative</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV %</th>
<th>NPV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>S&amp;S Isocode</td>
<td>125</td>
<td>11/13 (8.8%)</td>
<td>114/112 (91.2%)</td>
<td>86.66</td>
<td>100</td>
<td>100</td>
<td>98.2</td>
</tr>
<tr>
<td>Protein saver card 903</td>
<td>125</td>
<td>13/13 (10.4%)</td>
<td>112/112 (89.6%)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>FTA card</td>
<td>125</td>
<td>13/13 (10.4%)</td>
<td>112/112 (89.6%)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*PPV - Positive Predictive Value, NPV- Negative Predictive Value*

Figure 4.1 shows the Sensitivity, Specificity, Positive Predictive Value and Negative Predictive Value for the detection of *gag* gene of HIV-1 in whole blood specimen spotted on filter paper amplified by PCR method.
Figure 4.1 Comparison of results obtained from \textit{gag} gene using whole blood spotted on filter papers.

\begin{center}
\begin{tabular}{|c|c|c|c|}
\hline
& Sensitivity & Specificity & PPV & NPV \\
\hline
S&S Isocode & 90 & 100 & 95 & 90 \\
Proteinsaver card 903 & 85 & 100 & 90 & 85 \\
FTA card & 75 & 95 & 85 & 75 \\
\hline
\end{tabular}
\end{center}

\textit{PPV - Positive Predictive Value, NPV - Negative Predictive Value}
Figure 4.2 Detection of gag gene of HIV-1 by PCR for the whole blood specimen.

The positive bands of HIV-1 in infants were identified at 650 base pair using whole blood (Fig. 4.2).

The first lane is a 100 base pairs DNA marker.
The second and third lanes are positive and negative controls, respectively.
The lanes 4, 5 and 6 are infants’ positive results.
The lanes 7 and 8 are infants’ negative results.
Figure 4.3 Detection of gag gene of HIV-1 by PCR for the whole blood specimen spotted on filter paper.

The positive bands of HIV-1 in infants were identified at 650 base pair using protein saver card 903 (Fig. 4.3).

The first lane is a 100 base pairs DNA marker.

The second and third lanes are negative and positive controls respectively.

The lanes 4 and 6 are infants’ negative results.

The lanes 5, 7 and 8 are infants’ positive results.
4.4.3 Results obtained from whole blood spotted on filter papers using \textit{env} gene

DNA was extracted from whole blood spotted S&S Isocode stix filter paper and nested PCR was performed and the results were compared. Out of 13 HIV positive infants 8 were found to be HIV-1 positive (Table 4.3) and the discordant results were observed in five infant samples with the sensitivity being 72.2 %. The ages of these infants (3 males and 3 females) were between 2 weeks and 6 months. They were retested with another sample after a month’s period and similar results were observed. The concordant results were observed in all HIV-1 negative infants. The extracted DNA from whole blood spotted protein saver card 903 was used for nested PCR amplification and the results were compared with the whole blood results. Out 13 HIV positive infants, 12 were found to be HIV-1 positive (Table 4.3). The discordant result of one male infant was at 2 weeks of age and was retested at one month of the infants’ age and similar result was obtained. The results obtained from FTA card filter paper were similar to that of protein saver card 903 (Table 4.3). The discordant result was observed from the same infant and was retested along with protein saver card but similar result was observed. The sensitivity and the specificity of protein saver card and FTA filter papers were 92.85 % and 100 % respectively. The sensitivity of isocode filter paper was lesser when compared with protein saver card and FTA card.

Qizhang \textit{et al.} (2008) reported 100 % of specificity and 87.5 % of sensitivity by using protein saver card 903 for HIV exposed infants. Fischer \textit{et al.}
(2004) observed that the sensitivity was 100 % and the specificity was 98 %. The results of the present study indicate that protein saver card 903 and FTA card have similar sensitivity and specificity and the specificity results in particular are consistent with earlier reports (Qizhang et al., 2008; Fischer et al., 2004).

Table 4.3 Results of whole blood spotted on filter papers for the detection of \textit{env} gene in HIV-1 DNA by PCR.

<table>
<thead>
<tr>
<th>Specimen Source</th>
<th>Total no. of samples</th>
<th>HIV-1 Positive</th>
<th>HIV-1 Negative</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV %</th>
<th>NPV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>S&amp;S Isocode</td>
<td>125</td>
<td>8 /13 (6.4%)</td>
<td>117/112 (93.6%)</td>
<td>72.2</td>
<td>100</td>
<td>100</td>
<td>95.7</td>
</tr>
<tr>
<td>Protein saver card 903</td>
<td>125</td>
<td>12 /13 (9.6%)</td>
<td>113/112 (90.4%)</td>
<td>92.85</td>
<td>100</td>
<td>100</td>
<td>99.1</td>
</tr>
<tr>
<td>FTA card</td>
<td>125</td>
<td>12/13 (9.6%)</td>
<td>113/112 (90.4%)</td>
<td>92.85</td>
<td>100</td>
<td>100</td>
<td>99.1</td>
</tr>
</tbody>
</table>

\textit{PPV- Positive Predictive value, NPV- Negative predictive value}
Figure 4.4 shows the Sensitivity, Specificity, Positive Predictive Value and Negative Predictive Value for the detection of *env* gene of HIV-1 in whole blood specimen spotted on filter paper amplified by PCR method.

**Figure 4.4 Comparison of results obtained from *env* gene using whole blood spotted on filter papers.**

*PPV- Positive Predictive value, NPV- Negative predictive value*
The positive HIV-1 bands were detected at 700 base pairs in the gel picture (Fig 4.5) using env gene.

The first five lanes in the picture show positive results of infants’ samples.

The lanes of 6 and 7 are positive and negative controls respectively.

The last lane is a 100 base pairs DNA marker.
4.4.4 Results obtained from whole blood spotted on filter papers using pol gene

The results obtained using whole blood spotted S &S isocode filter paper are given in Table 4.4. Out of 13 HIV-1 positive infants, 3 samples were amplified and 10 infant samples could not be amplified. The age of these infants ranged from 2 weeks to 12 months and 6 were males and 4 were females. The specificity was 100 % and the sensitivity was 56.52 %, the positive and negative predictive values being 100 and 91.8% respectively. These infant samples were retested after 1 month and similar results were obtained. β-globin primers were amplified in all the discordant samples and it proved the presence of DNA in the filter paper. Using a protein saver card HIV-1 positive results were observed in 10 infants and 3 infant samples could not be amplified and these infant samples were retested after one month; similar results were obtained. This filter paper had 81.25 % of sensitivity and 100 % of specificity and the positive and negative predictive values were 100 and 97.4 % respectively. The results of protein saver card and FTA card were similar and the same 3 discordant samples were retested after one month; again it showed discordant results.

Bhavna et al. (2011) reported that the sensitivity and the specificity were 92.8% and 98.3% respectively. Ingrid et al. (2001) compared the sensitivity and the specificity between Washington children and Peru infants. The sensitivity of 98.4% and a specificity of 98.3% were observed in Washington study and the same study had a sensitivity of 99% and a specificity of 100% for Peruvian
The findings of 3 filter papers using pol gene showed 100 % of specificity and less sensitivity was observed with S&S isocode filter paper while the other two filter papers showed same sensitivity. The specificity of the present study agrees with earlier study’s result (Bhavna et al., 2011; Ingrid et al., 2001).

Table 4.4 Results of whole blood spotted on filter papers for the detection of pol gene in HIV-1 DNA by PCR.

<table>
<thead>
<tr>
<th>Specimen Source</th>
<th>Total no. of samples</th>
<th>HIV-1 Positive</th>
<th>HIV-1 Negative</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV %</th>
<th>NPV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>S&amp;S Isocode</td>
<td>125</td>
<td>3/13 (2.4%)</td>
<td>122/112 (97.6%)</td>
<td>56.52</td>
<td>100</td>
<td>100</td>
<td>91.8</td>
</tr>
<tr>
<td>Protein saver card 903</td>
<td>125</td>
<td>10/13 (8%)</td>
<td>115/112 (92%)</td>
<td>81.25</td>
<td>100</td>
<td>100</td>
<td>97.4</td>
</tr>
<tr>
<td>FTA card</td>
<td>125</td>
<td>10/13 (8%)</td>
<td>115/112 (92%)</td>
<td>81.25</td>
<td>100</td>
<td>100</td>
<td>97.4</td>
</tr>
</tbody>
</table>

PPV- Positive Predictive Value, NPV- Negative Predictive Value
Figure 4.6 shows the Sensitivity, Specificity, Positive Predictive Value and Negative Predictive Value for the detection of \textit{pol} gene of HIV-1 in whole blood specimen spotted on filter paper amplified by PCR method.

\textbf{Figure 4.6 Comparison of results obtained from \textit{pol} gene using whole blood spotted on filter papers.}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4_6.png}
\caption{PPV- Positive Predictive Value, NPV- Negative Predictive Value}
\end{figure}
Figure 4.7 Detection of *pol* gene of HIV-1 by PCR for the whole blood specimen spotted on filter papers.

The positive HIV-1 bands were detected at 665 base pairs in the gel picture (Fig. 4.7) using *pol* gene.

The first lane in the picture shows 100 base pairs DNA marker.

The 2-3 lanes are positive and negative controls respectively,

The lanes 4, 5 and 7 are infants’ negative results.

The lanes 6 and 8 lanes are infants’ positive results.
4.4.5 Roche Amplicor HIV-1 DNA

The results obtained from gag gene of nested PCR using whole blood were compared with the commercially available Roche Amplicor kit (Table 4.5) and based on the manufacturer’s instructions, the kit was designed to amplify gag gene only. HIV-1 DNA PCR test was performed using the kit and the results were compared and found to be 100 % concordant and showed 100 % of sensitivity, specificity, NPV and PPV for HIV exposed infants and controls. Thanyawee et al. (2003) found that 100 % of sensitivity, specificity, NPV and PPV in nested PCR using whole blood on comparing the results with Roche Amplicor kit in HIV-1 infant diagnosis and also concluded that the testing cost of nested PCR was less than Roche kit. The findings of the present study are in agreement with those of earlier study (Thanyawe et al., 2003).

Table 4.5 Comparison of results between nested PCR and Roche Amplicor kit

<table>
<thead>
<tr>
<th>No. of HIV-1 positive infants (n=13)</th>
<th>Age</th>
<th>Results of HIV-1 DNA PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nested PCR using gag gene</td>
</tr>
<tr>
<td>1</td>
<td>15 days</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>1 month</td>
<td>Positive</td>
</tr>
<tr>
<td>1</td>
<td>42 days</td>
<td>Positive</td>
</tr>
<tr>
<td>1</td>
<td>2 months</td>
<td>Positive</td>
</tr>
<tr>
<td>1</td>
<td>3 months</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>6 months</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>7 months</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>8 months</td>
<td>Positive</td>
</tr>
<tr>
<td>1</td>
<td>12 months</td>
<td>Positive</td>
</tr>
</tbody>
</table>
4.4.6 Comparison of results obtained from venipuncture blood and heel prick blood

Blood was obtained from venipuncture and heel prick of HIV-1 positive infants at two different times for reconfirmation of their HIV status. In the present study, two methods of blood collection were used to reconfirm their HIV status of HIV-1 positive infants and controls. The confirmation was done with protein saver card 903 and FTA card and 2 sets of filter paper were used for one infant. One set of filter paper was used for the blood obtained by venipuncture into EDTA tubes and then spotted. The other set of filter paper was used for heel prick of the infant by lancet and the blood was directly spotted on filter papers.

DNA was extracted from both the sets of blood spotted filter papers and nested PCR was performed using gag and env genes. The results were compared from these two methods of blood collection and it was found to be 100 % concordant for using gag gene and one sample could not be amplified in env gene. The sensitivity and the specificity were 100 % using gag gene and 92.85 % of sensitivity and 100 % of specificity were obtained using env gene in protein saver card and FTA card filter papers.

Goulder et al. (2008) reported that using gag gene was more conserved than env gene in infant diagnosis. Mehta et al. (2010) also concluded that gag region was a conserved region to identify the HIV status of the infants. The obtained results have indicated that gag gene is found to be more conserved region than env and pol genes for HIV-1 infant diagnosis.
In conclusion, the present investigation provides valuable information regarding the use of HIV-1 DNA PCR of whole blood collected on filter papers for infant testing of HIV-1 infection. The filter papers require less volume of blood sample for DNA extraction than the whole blood sample assay procedures; two punches of 3 mm were enough to perform the DNA extraction and each 3-mm-diameter filter paper disc contained approximately 5 μl of whole blood (Denny et al., 1992). Ingrid et al. (2001) reported that even for a low copy number of HIV-1 infection could be amplified using FTA filter paper.

In the present study, the occurrence of false negative results may be due to the presence of less copies of virus in the infants. The filter papers were checked for the presence of DNA after the extraction from each filter paper using β-globin primers and it was found that all the samples were free of PCR inhibitors, as β-globin was amplified in all of the specimens. DNA extracted from S&S Isocode stix filter paper showed failure of amplification in many samples and the sensitivity was found to be less when compared to protein saver card 903 and FTA card filter papers. The present findings indicate that protein saver card 903 and FTA card filter papers have good sensitivity and specificity and can be used in rural areas for the adsorption of whole blood to filter papers which provide an alternate resource for HIV-1 infant diagnosis in resource limited settings.

The findings clearly indicate that either whole blood coated on filter papers or blood coated directly by heel prick are simple, sensitive, and specific test appropriate for the diagnosis of HIV-1 infection in infants. It can be suggested
that filter papers are very useful for sample collection device in HIV-1 infant diagnosis in resource limited settings and the blood which is directly coated by heel prick is more convenient than venipuncture for the health care workers.

In addition, filter paper methods require less equipment and less technical expertise besides being less expensive and carrying less of a biohazard risk than whole blood samples and require minimal storage facilities, since the samples are stable at room temperature for prolonged periods and are easy to ship, facilitating centralized laboratory testing centers. Chung et al. (2004) reported that filter paper was able to store HIV-1 DNA for more than 4.2 years and the decaying of the sample was very minimal and the blood coated onto this filter paper was very useful for future studies. Caroline et al. (2008) observed that HIV-1 DNA stability at room temperature on whole blood spotted protein saver card 903 filter paper was up to 15 weeks and on FTA filter paper was up to 4 years. The cost of protein saver card 903 was cheaper than FTA filter paper.

The blood collected on filter paper provides a simple approach for HIV-1 diagnosis in infants. HIV-exposed infants need regular follow-up since they are at the risk of morbidity and mortality regardless of infection status.

Key findings of the present study using the blood spotted filter papers:

- A more suitable and potentially cost-effective molecular testing method incorporating PCR has been identified. It involves the detection of HIV-1DNA, using blood spotted filter papers.
The study has demonstrated the reliability of a cost-effective DNA-based infant HIV-1 test based on analysis of whole blood spotted on filter papers. HIV-1 was detected with high sensitivity using whole blood spotted filter papers. This suggests that this assay method can provide an alternative cost-effective, reliable and rapid method for early detection of HIV-1 infection in infants.

4.5 Biochemical findings
4.5.1 Effect of nevirapine on the activity of liver marker enzymes

4.5.1.1 Infants

Several antiretroviral regimens have been shown to reduce mother-to-child transmission of HIV. In developing countries the most practical regimen is the use of a single oral dose of nevirapine to the mother at the start of labor and to the infant shortly after birth. The present study is to evaluate the abnormalities associated with nevirapine in liver marker enzymes in HIV exposed infants and their mothers treated with nevirapine.

The results concerning the variables of Total bilirubin, AST, ALT and ALP for HIV exposed infants and controls are presented in Table 4.6. Analysis of these variables demonstrated no significant differences to be observed regarding total bilirubin, AST and ALP ($p > 0.05$) among HIV exposed infants and controls. Analysis of ALT activities showed significantly higher ($p < 0.05$) in HIV exposed infants than controls. Elevated ALT activities were observed in 16.8% (n=21) of
HIV exposed infants and they were in the age group of 3-6 months and 4 of them were HIV-1 infected and 17 were HIV-1 negative and no one had the symptoms of jaundice in both the groups. To estimate severity, increased activities of ALT in infants were graded based on Pediatric Toxicity tables, and found to be in the mild category (grade 1 level). None of the infants developed toxicity more severe than grade 1 level.

Patrícia et al. (2007) reported that no significant differences were observed in total bilirubin, AST, ALT and ALP between HIV exposed and control infants. Silverman et al. (1998) observed that activities of AST were higher in 58% of the infants. Taha et al. (2002) reported that the infants at birth and after 6 weeks of age, mean ALT activities were higher among infants than controls and the increased ALT values were mild-grade 1 level. The data obtained from the present study were very well supported by Taha’s (2002) study.

The follow-up is important for these infants to maintain the normal levels of the ALT enzyme activity. Higher levels of ALT were suggestive of acute liver damage due to exposure to hepatotoxic agents. At birth ALT activities are higher, and subsequently decline to adult levels at approximately 3 months of age (Murray, 1984). From the present data, it can be suggested that even short-term antiretroviral drugs could influence the levels of ALT. Stratifying these findings by HIV status of the infant, suggests that these increases could be due in part to
immunological changes which accompany HIV infection because levels of ALT are highest among infected infants.

Table 4.6 Effect of nevirapine on liver marker enzymes and total bilirubin among HIV exposed infants and controls

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HIV exposed infants</th>
<th>Controls</th>
<th>p -value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (IU/L)</td>
<td>12.1±6.16</td>
<td>13.9 ±5.61</td>
<td>0.145^</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>31.5 ± 17.57</td>
<td>13.45 ± 4.76</td>
<td>0.020*</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>385.5 ± 105.6</td>
<td>372.6 ± 79.4</td>
<td>0.532^</td>
</tr>
<tr>
<td>Total Bilirubin</td>
<td>0.7 ± 0.29</td>
<td>0.79 ± 0.25</td>
<td>0.144^</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SD.
*P < 0.05 – Statistically Significant compared with control group
^ Non significant compared with control group
Figure 4.8 show the effect of Nevirapine on ALT among HIV exposed infants and controls. Each point represents Mean ± SD of five samples; * indicates Statistical significant (*P < 0.05) difference among HIV exposed infants and controls.

**Figure 4.8 ALT levels of infants**

![Graph showing ALT levels of infants](image)

*P < 0.05 – Statistically Significant compared with control group

### 4.5.1.2 Mothers

Nevirapine may cause severe or life-threatening liver toxicity, usually emerging in the first six weeks of treatment. The extent of liver damage is assessed by evaluating the levels of marker enzymes. The activities of the marker enzymes such as AST, ALT, ALP and GGT are summarized. The results of total
bilirubin, AST, ALT, ALP, GGT, total protein and albumin for HIV positive study mothers are presented in Table 4.7. There were no significant differences in total bilirubin and albumin ($p > 0.05$) to be observed. Total protein was found to be significantly lower in HIV positive mothers ($p < 0.05$) than normal control mothers. The activities of the enzymes-AST, ALP and GGT were significantly higher in HIV positive mothers than the controls. The elevation of AST activities was observed in 4% of HIV positive mothers, (mean= 57.4 IU/L) which was more than 1.25 times the upper limit normal (ULN). Increased ALT activities were found out in 10.4% of HIV positive mothers, the mean being 61.38 IU/L, which was 1.5 times higher than the upper limit normal level. Elevations of both AST and ALT activities were detected in 13.6% of HIV positive mothers, the mean level for AST and ALT activities being 66.23 and 78.05 IU/L respectively. (1.5 and 1.7 times higher than ULN) and the elevated enzyme levels were observed between 23 and 45 days after initiation of nevirapine drug, except one mother who had elevations of both these enzymes at 8 months after the exposure of nevirapine. The activities of ALP and GGT were found to be in one HIV positive mother after 5 months of nevirapine intake. To estimate severity, increased activities of AST and ALT in HIV positive mothers were graded based on Toxicity grading scale, the elevations were in the mild category -grade 1 level and none of the mothers developed toxicity more severe than grade 1 level.

William et al. (2007) observed that the elevations of AST and ALT activities were found in less than 7 weeks of nevirapine initiation. Wooltorton,
(2004) reported that the asymptomatic minor elevations in liver enzymes were common and they occurred during the first six weeks. The findings of the present study are well supported by the earlier studies (William et al., 2007; Wooltorton, 2004) and no hepatotoxicity was observed in any of HIV positive mothers and controls. Based on adult antiretroviral guidelines the activities of AST and ALT should be measured at baseline, every 2 weeks for the first month, monthly through 4 months, and every 1 to 3 months thereafter (Adult Antiretroviral Guidelines, www.aidsinfo.nih.gov.)

Table 4.7 Effect of nevirapine on liver function profile among HIV positive mothers and controls

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HIV Positive mothers</th>
<th>Controls</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Bilirubin (mg/dl)</td>
<td>0.65 ± 0.25</td>
<td>0.620 ± 0.19</td>
<td>0.434^</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>32.5 ± 17.57</td>
<td>21.1 ± 6.31</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>38.88 ± 21.65</td>
<td>24.03 ± 5.59</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>164.3 ± 30.06</td>
<td>145.50 ± 32.92</td>
<td>0.004**</td>
</tr>
<tr>
<td>GGT (IU/L)</td>
<td>20.05 ± 8.95</td>
<td>14.07 ± 5.22</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Total Protein (gm/dl)</td>
<td>7.16 ± 0.68</td>
<td>7.43 ± 0.57</td>
<td>0.048*</td>
</tr>
<tr>
<td>Albumin (gm/dl)</td>
<td>3.96 ± 0.59</td>
<td>4.07 ± 0.38</td>
<td>0.210^</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SD

***P < 0.001, **P < 0.01, *P < 0.05 – Statistically Significant compared with control group

^ Non significant compared with control group
Figure 4.9 indicates the effect of Nevirapine on liver marker enzymes, total protein and albumin among HIV positive mothers and controls. Each point represents Mean ± SD of five samples; * indicates Statistical significant (**p < 0.01, *p < 0.05) difference among HIV positive mothers and controls.

**Figure 4.9 Liver function profiles of mothers**

***P < 0.001, **P < 0.01, *P < 0.05 – Statistically Significant compared with control group***
Figure 4.10 shows the effect of nevirapine on total bilirubin among control and HIV positive mothers. Each point represents Mean ± SD of five samples.

*Figure 4.10 Total bilirubin levels of mothers*

![Bar chart showing total bilirubin levels of mothers](image)

^Non significant compared with control group
4.6 Hematological and Immunological findings

4.6.1 Infants

Hematological parameters are affected by many factors, including age, sex, diet, recent nutritional status, and consumption of medications or illicit drugs. Infants’ hemoglobin and hematocrit levels may be influenced by many factors, including the mode of delivery. Anemia can occur at any phase of HIV infection and its prevalence and severity are correlated with progression of the disease.

The results of hemoglobin, hematocrit, white blood cell and total lymphocyte count were performed in HIV exposed infants and HIV non exposed infants are given in Table 4.8. There was a significant reduction in hemoglobin levels, hematocrit, white blood cell count and total lymphocyte counts for HIV exposed infants rather than in controls ($p < 0.001$ and $p = 0.001$ respectively). Significant decrease in CD4 and CD8 counts were observed in HIV exposed infants rather than in controls.

The hematological data of the present study was graded according to DMID table which was useful to identify the severity of the disease levels. Out of 125 HIV exposed infants 28 % had less than 10 gm/dl of hemoglobin, (mean =8.914 g/dl) 20 were male infants and 15 were female infants. 17 infants were in grade 1 level, 2 female infants of grade 1 level were found to be HIV-1 positive; they were 6 and 10 months old, the other 15 were found to be HIV-1 negative. In grade 2
level (n=17) 2 infants were found to be HIV-1 positive, one male and one female, their ages were 9 months and 42 days; one female infant was in grade 3 level whose age was 2 weeks and found to be HIV-1 Positive.

Feiterna et al. (2007) reported that anemia was found in 53.8 % of HIV exposed infants and was in grade 2 level during the first 3 months of life. Anita et al. (2009) revealed that 66 % of the HIV infected children had anemia and 8 % had severe anemia. Woong et al. (2008) suggested that there was no increased risk of severe anemia in both HIV exposed and non exposed infants. Kasonde et al. (2009) have found that hemoglobin levels, hematocrit, white blood cell counts were lower among HIV-infected infants than in uninfected infants. Landreau et al. (2002) revealed that lower hemoglobin levels existed in HIV exposed infants. Bunders et al. (2005) reported that the CD4 cell count was lower only during the first year of life, but the CD8 count was reduced through 8 years of life. Moodley et al. (1997) reported that CD4/CD8 ratio was a good predictor of poor clinical outcome at 3 months and the authors concluded that CD4/CD8 ratio and %CD4 among lymphocytes are reliable markers of HIV-1 infection in pediatric population. Savita et al. (2008) observed that CD4 T cell counts of HIV-infected infants decrease faster than those of uninfected infants.

It is observed from the present study that 28% of HIV exposed infants had developed anemia and none of these infants had developed severe anemia, there was no significant association in anemia and gender of HIV exposed infants. The
findings of the present findings are well in accord with the results of earlier studies (Kasonde et al., 2009; Landreau et al., 2002).

Treatment of anemia should be towards correcting the underlying cause with iron supplementation for iron deficiency and control of opportunistic infections in infants. Hematological measurements can also be useful for clinical monitoring of HIV-infected individuals when viral load testing and CD4 cell count monitoring are not readily available.

Table 4.8 Hematological and Immunological findings in HIV exposed infants and controls

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HIV exposed infants</th>
<th>Controls</th>
<th>$p$ - value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>10.95 ± 1.57</td>
<td>12.62 ± 0.93</td>
<td>&lt; 0.001***</td>
</tr>
<tr>
<td>Haemotocrit (%)</td>
<td>33.59± 4.01</td>
<td>38.73 ± 4.78</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>WBC (cells/mm$^3$)</td>
<td>7246.40 ± 2513.88</td>
<td>9226.67 ± 1558.72</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>TLC (cells/mm$^3$)</td>
<td>2800.00 ± 632.74</td>
<td>3346.67 ± 792.52</td>
<td>0.001**</td>
</tr>
<tr>
<td>CD4 (cells/mm$^3$)</td>
<td>612.58±218.19</td>
<td>909.20±119.81</td>
<td>0.005**</td>
</tr>
<tr>
<td>CD8 (cells/mm$^3$)</td>
<td>1005.50±242.64</td>
<td>1212.80±150.71</td>
<td>0.034*</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>0.60±0.14</td>
<td>0.75±0.19</td>
<td>&lt;0.001***</td>
</tr>
</tbody>
</table>

Values are expressed as Mean± SD.

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ – Statistically Significant compared with control group
Figure 4.11 indicates the hemoglobin levels among HIV exposed infants and controls. Each point represents Mean ± SD of five samples; * indicates Statistical significant (***) $p < 0.001$ difference among HIV exposed infants and controls.

**Figure 4.11 Hemoglobin levels of infants**

*** $P < 0.001$ Statistically Significant compared with control group
Figure 4.12 show the percentage of hematocrit among HIV exposed infants and controls. Each point represents Mean ± SD of five samples; * indicates Statistical significant (**p < 0.001) difference among HIV exposed infants and controls.

Figure 4.12 Hematocrit levels of infants

*** P < 0.001 Statistically Significant compared with control group
Figure 4.13 indicates the abnormalities of hematological and immunological findings among HIV exposed infants and controls. Each point represents Mean ± SD of five samples; * indicates Statistical significant (*** \( p < 0.001 \), ** \( p < 0.01 \), * \( p < 0.05 \)) difference among HIV exposed infants and controls.

**Figure 4.13 Hematological and Immunological findings among infants**

![Graph showing hematological and immunological findings](image)

*** \( P < 0.001 \), ** \( P < 0.01 \), * \( P < 0.05 \) – Statistically Significant compared with control group
Table 4.9 Results from multivariate logistic regression for infants’ findings associated with HIV status

<table>
<thead>
<tr>
<th>Parameter (cells/mm³)</th>
<th>OR</th>
<th>95% CI for OR</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>0.994</td>
<td>0.991, 0.999</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>CD8</td>
<td>1.003</td>
<td>1.001, 1.006</td>
<td>&lt;0.001***</td>
</tr>
</tbody>
</table>

*** P < 0.001 - Statistically significant compared with control group

Dependent variable: HIV status of infants

OR- Odds Ratio, CI- Confidence interval

All the infant’s findings significantly associated with HIV-1 status of the infants in the univariate analysis were considered for multivariate analysis. Multivariate logistic regression was used to find factors independently associated with HIV-1 status of infants. The factors which were highly associated were excluded to avoid multicollinearity. CD4 and CD8 were independently associated with infants’ HIV status and were significantly less for HIV-1 positive infants.

The results of liver marker enzymes and hematological findings (Table 4.10) of nevirapine treated HIV-1 exposed infants (HIV-1 positive and negative infants) were compared and no significant differences were observed.

Diana et al. (2010) have shown that higher levels of WBC, TLC and lower levels of hemoglobin were observed in HIV-1 positive infants than negative infants. Taha et al. (2002) found that no significant difference
between the nevirapine treated HIV-1 exposed infants in liver and hematological parameters existed. The findings of the present study agree well with Taha’s (2002) findings and have shown that maternal HIV-1 disease and nevirapine exposure do not influence infants’ liver and hematological findings.

Table 4.10  Biochemical and hematological findings among HIV exposed infants

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HIV-1 Positive infants (n=13)</th>
<th>HIV-1 Negative infants (n=112)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Bilirubin (mg/dl)</td>
<td>0.75±0.25</td>
<td>0.698±0.30</td>
<td>0.523^</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>14.08±7.65</td>
<td>11.87±5.96</td>
<td>0.222^</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>11.54±5.39</td>
<td>10.35±4.83</td>
<td>0.392^</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>416.23±150.71</td>
<td>381.96±99.42</td>
<td>0.270^</td>
</tr>
<tr>
<td>Hemoglobin (gm/dl)</td>
<td>10.55±1.65</td>
<td>11.0±1.56</td>
<td>0.324^</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>32.215±3.73</td>
<td>33.754±4.03</td>
<td>0.192^</td>
</tr>
<tr>
<td>TLC (cells/mm³)</td>
<td>2438.46±1809.48</td>
<td>2842.34±1614.45</td>
<td>0.204^</td>
</tr>
<tr>
<td>WBC (cells/mm³)</td>
<td>6653.85±768.15</td>
<td>7315.18±2487.06</td>
<td>0.371^</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SD
^ Non significant compared with controls

4.6.2 Mothers

Nutrition has been acknowledged as an important factor in the course of HIV infection and it is generally accepted as a major determinant of immune functioning. Nutritional factors, although not the most important etiological
determinants may change immune function so as to facilitate disease progression, influence viral expression, and play a significant role in disease related morbidity and mortality. The total lymphocyte count can be used as a surrogate marker for CD4 cell count to guide antiretroviral therapy initiation. There was a significant decrease ($p < 0.001$) in all hematological and immunological parameters of HIV positive mothers rather than normal control mothers (Table 4.11).

In the present study 83.2% of HIV positive mothers were found to be more anemic (hemoglobin <12.0 gm/dl) than HIV negative mothers (41.66%). The severity of the anemia levels was calculated using Toxicity grading table: 23.2% of HIV positive mothers were in grade 1 level (11-12 gm/dl of hemoglobin), 42.4% were in grade 2 (9.5-10.9 gm/dl of hemoglobin), 14.4% were in grade 3 (8-9.4 gm/dl of hemoglobin) and 3.2% were in grade 4 (< 8.0 gm/dl of hemoglobin). Among the control mothers 18% were in grade 1 level and none of them were in grade 2, 3 and 4 levels. Neutropenia (white blood count was <2000 cells/mm$^3$) was observed in 2/125 (1.6%) of HIV positive mothers.

The positive correlation between CD4 and total lymphocyte counts was observed in 3/125 mothers who had < 200 cells/mm$^3$ of CD4 counts and < 2000 cells/mm$^3$ of total lymphocyte counts.

Papathakis et al. (2007) reported that anemia was found to be higher in HIV positive mothers than HIV negative mothers. Moyle, (2002) have reported that
anemia is a prognostic marker of future disease progression or death, independent of CD4 and viral load. Elisaphane et al. (2012) also explained that anemia was more common in HIV positive women than in HIV negative women and also found out that neutropenia was more common in HIV positive women than in HIV negative women. Erhabor et al. (2005) observed 24% of neutropenia in HIV positive women. Adaobi et al. (2012) reported that HIV infection lowered the hematocrit in HIV positive women and was directly proportional to CD4 counts. Hematological abnormalities such as anemia and neutropenia are commonly observed in patients infected with HIV (Ballem et al., 1992).

Deresse et al. (2008) found out that total lymphocyte count was lower in HIV positive mothers than in HIV negative mothers. Jacob et al. (2011) also observed lower total lymphocyte count in HIV positive mothers. Victor et al. (2008) found lower CD4 and CD8 cell counts in HIV positive women. Gupta et al. (2007) reported that antenatal and postpartum women in resource-limited settings need to be tested for CD4 cell counts instead of relying on TLC alone and also suggested that when CD4 cell counts are unavailable, TLC should be used combined with WHO’s staging. Stebbing et al. (2005) also indicated that despite minimally less reliability of TLC as a surrogate for CD4 it could be used. The guidelines from the WHO acknowledged that TLC may be used to make treatment decisions in resource-poor counties where CD4 counts are not available when patients are mildly symptomatic. For adults with Stage II illness, TLC threshold
for initiating antiretroviral therapy is 1,200 cells/mm$^3$ (www.who.int/3by5/ARVmeetingreport_June2005).

Anemia has been associated with HIV disease progression and an increased risk of death. Nutritional deficiencies may develop during any stage in the HIV infected individual. Anemia of chronic disease seems evident in a large percentage of HIV positive women. Hemoglobin concentration could also be used as a reliable biomarker of the prognosis in HIV-infected patients. Based on WHO guidelines 15 mothers included in the present study were eligible for antiretroviral therapy whose CD4 counts were < 350 cells/mm$^3$.

Table 4.11 Hematological and Immunological findings among HIV positive mothers and controls

<table>
<thead>
<tr>
<th>Variables</th>
<th>HIV positive mothers</th>
<th>Controls</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (gm/dl)</td>
<td>10.65± 1.33</td>
<td>12.13±0.74</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>32.16±3.97</td>
<td>36.38±2.20</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>WBC (cells/mm$^3$)</td>
<td>5399.20 ± 1999.09</td>
<td>8653.33 ± 1442.39</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>TLC (cells/mm$^3$)</td>
<td>1502.40 ± 643.010</td>
<td>3123.33 ±581.130</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>CD4 (cells/mm$^3$)</td>
<td>494.30 ±176.402</td>
<td>964.60 ±67.130</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>CD8 (cells/mm$^3$)</td>
<td>1091.10 ±131.91</td>
<td>1127.96 ± 281.29</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>0.46±0.16</td>
<td>0.81±0.07</td>
<td>&lt;0.001***</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SD

*** $P < 0.001$ – Statistically Significant compared with controls
Figure 4.14 indicates the hemoglobin levels among HIV positive mothers and controls. Each point represents Mean ± SD of five samples; * indicates Statistical significant (*** $p < 0.001$) difference among HIV positive mothers and controls.

*** $P < 0.001$ – Statistically Significant compared with controls
Figure 4.15 shows the percentage of hematocrit among HIV positive mothers and controls. Each point represents Mean ± SD of five samples; * indicates Statistical significant (**p < 0.001) difference among HIV positive mothers and controls

*** P < 0.001 – Statistically Significant compared with controls
Figure 4.16 indicates the abnormalities of hematological and immunological findings among HIV positive mothers and controls. Each point represents Mean ± SD of five samples; * indicates Statistical significant (** p < 0.01, * p < 0.05) difference among HIV positive mothers and controls.

*** P < 0.001 – Statistically Significant compared with controls
Table 4.12 Results from multivariate logistic regression for mothers’ findings associated with HIV status

<table>
<thead>
<tr>
<th>Parameters</th>
<th>OR</th>
<th>95% CI for OR</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (IU/L)</td>
<td>1.102</td>
<td>0.99, 1.24</td>
<td>0.089^</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>0.01</td>
<td>0.01, 0.01</td>
<td>&lt;0.001***</td>
</tr>
</tbody>
</table>

***P < 0.001,– Statistically Significant compared with controls
^ Non significant compared with controls
Dependent variable: HIV status of mothers
OR- Odds Ratio, CI-Confidence interval

All the mothers’ findings significantly associated with mothers’ HIV status in the univariate analysis were considered for multivariate analysis. Multivariate logistic regression was used to find factors independently associated with mothers’ HIV status. The factors which were highly correlated were excluded in the multivariate analysis to avoid multicollinearity. AST and CD4/CD8 ratio were independently associated with mothers’ HIV status. CD4/CD8 ratio was found to be significantly lower in mothers.
4.7 Comparisons of biochemical and hematological findings based on
CD4 levels among HIV positive mothers

The results of liver marker enzyme activities and hematological findings
obtained from HIV positive mothers were divided into 2 groups on the basis of
CD4 cell counts (<350 and >350 cells/mm$^3$) (Table 4.13). Significant difference
was found in the levels of hemoglobin ($p=0.035$), which was found to be lower in
mothers having < 350 cell counts than those having >350 cell counts. There was
no significant difference in liver and other hematological findings. Elisaphane et
al. (2012) indicated that lower hemoglobin levels were found in women who had
lower CD4 cell counts of < 350 cells. Gil et al. (2011) found an association
between CD4 count and hemoglobin level and hemoglobin concentration was
strongly correlated with death rates for patients with both low and high CD4
counts. The present results are consistent with earlier reports (Elisaphane et al.,
2012 ; Gil et al., 2011). Anemia was more common in the HIV positive mothers,
especially those with greater disease progression as indicated by lower CD4 cell
counts. Anemia in HIV-infected persons is associated with CD4 cell depletion and
progression to AIDS and is one of the strongest predictors of HIV mortality and
poor responses to antiretroviral therapy. The use of hemoglobin levels as a
predictor factor for the prognosis, especially in countries with limited resources
where routine enumeration of CD4 cells may not be feasible.
Table 4.13 Biochemical and hematological findings based on CD4 cells among HIV positive mothers

<table>
<thead>
<tr>
<th>Variables</th>
<th>HIV Positive mothers</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1</td>
<td>Group 2</td>
</tr>
<tr>
<td></td>
<td>CD4 &lt; 350 cells/mm³</td>
<td>CD4 &gt; 350 cells/mm³</td>
</tr>
<tr>
<td>Age</td>
<td>26.71±3.22</td>
<td>25.05±3.58</td>
</tr>
<tr>
<td>Total Bilirubin (mg/dl)</td>
<td>0.72±0.256</td>
<td>0.64 ±0.25</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>34.71±17.68</td>
<td>32.22 ±17.62</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>46.00±21.144</td>
<td>37.98 ±21.64</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>150.36±28.99</td>
<td>144.88 ±33.45</td>
</tr>
<tr>
<td>GGT (IU/L)</td>
<td>19.79±7.758</td>
<td>20.08±9.12</td>
</tr>
<tr>
<td>Total Protein (gm/dl)</td>
<td>7.107±0.6486</td>
<td>7.16±0.69</td>
</tr>
<tr>
<td>Albumin (gm/dl)</td>
<td>3.893±0.5890</td>
<td>3.96 ±0.59</td>
</tr>
<tr>
<td>Hemoglobin (gm/dl)</td>
<td>9.95±1.00</td>
<td>10.74±1.34</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>30.31±3.86</td>
<td>32.39± 3.93</td>
</tr>
<tr>
<td>WBC (cells/m³)</td>
<td>5685.71±1754.492</td>
<td>5363.06±2032.10</td>
</tr>
<tr>
<td>TLC (cells/mm³)</td>
<td>1278.57±678.435</td>
<td>1530.63±636.009</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SD
*P < 0.05 – Statistically Significant compared with group1 and 2
^ Non significant compared with group 1 and 2
Figure 4.17 shows the levels of hemoglobin among group1 and group2 of HIV positive mothers. Each point represents Mean ± SD of five samples; * indicates Statistical significant (*p < 0.05) difference among HIV positive mothers based on their CD4 cell counts.

Figure 4.17 Hemoglobin levels based on CD4 cells among HIV positive mothers

![Graph showing hemoglobin levels based on CD4 cells among HIV positive mothers.](image)

*P < 0.05 – Statistically Significant compared with group1 and 2
Table 4.14 Results from multivariate logistic regression for mother’s findings associated with mothers’ CD4 value < or >= 350 cells

<table>
<thead>
<tr>
<th>Parameter</th>
<th>OR</th>
<th>95% CI for OR</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>1.58</td>
<td>1.02, 2.44</td>
<td>0.04*</td>
</tr>
</tbody>
</table>

*P < 0.05 – Statistically Significant compared with controls

Dependent variable: mothers CD4 < or >= 350 cells

OR- Odds Ratio
CI-Confidence Interval

All the mothers’ findings significantly associated with mothers CD4 cells in the univariate analysis were considered for multivariate analysis. Multivariate logistic regression was used to find factors independently associated with CD4 cells of HIV positive mothers. Those factors which were highly correlated were excluded to avoid multicollinearity. Mother’s hemoglobin was independently associated with mothers CD4 cells. Hemoglobin was significantly high for mothers with CD4 cells more than 350 cells/mm³ than the mothers with < 350 cells/mm³.

4.8 Quantitative HIV-1 RNA PCR

4.8.1 Correlation of CD4 and viral load

Viral load is an important predictor of disease progression in HIV-infected adults. The combined use of CD4 cell counts and viral load testing will provide a more complete picture of a person’s risk of disease progression and response to therapy. CD4 cell counts indicate the status of an individual’s immune system and
viral load tests indicate the activity of the virus. Having a low CD4 count and high viral load has been established to be a risk factor for progression to more serious HIV disease. In the present study, quantification of HIV-1 RNA viral load was performed for 20 HIV positive mothers (mean age = 26 years). It was observed that viral load levels of above 10,000 copies/ml were found in 15 mothers who had <350 cells of CD4 counts, the mean viral load being 20,935.35 copies/ml and the mean CD4 cell counts being 328.6 cells/mm$^3$. HIV-1 RNA viral load assay was performed for all the 13 HIV-1 positive infants. In 11 infants the viral load could not be detected (< 400 copies/ml) and 2 infants had 767 and 680 copies/ml of viral load, both were female infants and the age was 6 months and 42 days respectively. Both these 2 infants’ mothers had higher viral load (> 10,000 copies /ml). There was no significant difference between mothers’ viral load and their infants’ CD4 cell counts ($p=0.351$).

Alexandra et al. (2007) observed that as long as the viral load levels were 10,000 copies/ml, the CD4 cell counts were stable and the risk of clinical progression was low. Andrew et al. (2010) found out that 96% of patients were with a CD4 cell count below 200 cells/mm$^3$ and had a viral load of above 10,000 copies/ml. In addition, 86% of those whose CD4 cell count fell to the dangerously low level of 50 cells/mm$^3$ had a viral load above 50,100 copies/ml and they also observed that there was no association between age and changes in viral load. Mofenson et al. (1997) reported that HIV-1 RNA load in infancy was predictive of
rapid progression to AIDS or death. PENTA study, (1998) reported that the relationship between HIV-1 RNA and CD4 cell count appeared to change with age, becoming inverted only after 2 years of the infants’ age.

The investigations are observed from the present study that CD4 cell counts and viral loads had strong inverse correlation \( (p \leq 0.001; r = -0.728) \), as the CD4 count increased, viral load decreased (Fig 4.18). There was no significant association between the age and viral load for the mothers. Either CD4 counts or viral loads are predictive of the benefits of antiretroviral therapy and the findings are in accord with earlier study. These factors taken together may provide a better way to know when to start or change therapies than with the earlier method of using only CD4 levels.
Figure 4.18 Scatter diagram of CD4 and viral load for HIV positive mothers

Values for CD4 cell counts are expressed as cells/mm$^3$ and the viral loads are expressed as copies/ml.

4.9 Risk factors for HIV-1 infection through breast feeding

Mother-to-child transmission of HIV-1 can occur in utero, during delivery, and postnatally through breastfeeding (Kourtis et al., 2001). Breastfeeding is responsible for a high proportion of mother-to-child transmission in developing
countries. In this study, out of 125 mothers 13 (10.4 %) were transmitted HIV infection to their infants. Out of 13 HIV-1 positive infants, 9 were breastfed, three were found to be HIV-1 positive who had breastfed exclusively for 5 months and six were given mixed feeding (breast milk and bottle feeding) and their mothers weaned between 4-6 months. The rate of HIV transmission is higher in infants who had received mixed feeding rather than exclusive breastfeeding.

The remaining 4 HIV positive infants were not breastfed by mothers. One infant was found to be HIV-1 positive at 15 days of age; the other 2 infants were at 1 month and 1 at 42 days of life. The infection could have occurred either in utero or during labour (intrapartum). Magder et al. (2005) explained that in utero HIV-1 infection could be defined as an infant with the first positive HIV-1 DNA PCR assay at 7 days of age or younger; intrapartum infection could be defined the first positive PCR assay after 7 days of age.

Thomas (2007) observed that infants who had received mixed feeding during their first six months were at higher risk of HIV infection than those who had been breast-fed exclusively and also found that transmission risk was associated with maternal CD4 cell counts of 200–500 cells /mm$^3$ had a higher risk of HIV infection to the infants. Jenifer (2004) reported that lower maternal CD4 cell counts possibly reflected higher virus loads in maternal blood and breast milk, both of which were associated with a higher risk of mother-to-child transmission of HIV-1 and a higher plasma virus load was associated with a higher probability
of transmission through breast milk. Leroy *et al.* (2003) also reported that the risk of postnatal infection was associated with high maternal viral load. Exclusive breastfeeding, in which the infant receives no supplemental fluids or foods apart from breast milk during the first 6 months of life, is associated with a lower risk of HIV transmission from breast milk when compared with mixed feedings (Iliff *et al.*, 2005; Coovadia *et al.*, 2007).

The findings of the present study is well supported by the earlier studies (Thomas, 2007; Jenifer, 2004; Leroy *et al.*, 2003) and found out that the 13 transmitted (both breastfed and non breastfed) mothers had high viral load (> 10,000 copies/ml) and low CD4 cell counts (< 350 cells/mm$^3$).

WHO (2008) released new recommendations on infant feeding by HIV-positive mothers, for the first time requiring the HIV-positive mothers or their infants to take antiretroviral drugs throughout the period of breastfeeding and until the infant is 12 months old. This means that the child can benefit from breastfeeding with very little risk of getting infected with HIV.

WHO also recommends that all mothers, regardless of their HIV status, should practice exclusive breastfeeding – which means no other liquids or food should be given for the first six months. After six months, the baby should start on complementary foods (http://www.who.int).
4.10 Nevirapine resistance determination by Oligonucleotide ligation assay

Increasing HIV RNA levels may indicate the development of drug resistance and HIV RNA may give an earlier warning of impending drug failure, signals that can be read before a person suffers serious decline of CD4 cell counts, disease progression or death. Nevirapine resistance mutations were detected for 5/125 (4%) of HIV positive mothers and 120 (96%) mothers had not developed mutations. The time exposure of single dose nevirapine among these 5 mothers was between 4 and 8 weeks. K103N mutation was found in 4 mothers and Y181C mutation was found in 1 mother and none of their HIV-1 positive infants (n=13) had developed any mutations.

Mark et al. (2010) observed that nevirapine resistant at < 8 weeks of age, the rapid decay of nevirapine-resistant HIV-1 among infants was parallel to the decay in post-partum women. Morris et al. (2004) have found that 35% of women had nevirapine resistance mutations which were detected in 7 weeks after the administration of single dose nevirapine. Thor et al. (2010) found that mutations occurred in 33% of the women and the median time was 45 weeks after single dose nevirapine therapy, by OLA. Eshleman et al. (2001) have observed the nevirapine resistant in 6-8 weeks after delivery in 19% women treated with a single dose nevirapine. Jacob et al. (2011) have found out that nevirapine-resistance mutations occurred in 28% of women at 6 weeks after delivery. The lysine (K) to asparagine (N) mutation at codon 103 (K103N) was the most common mutation in their
study. In another study, drug resistance mutations were observed in 33% patients at one month after nevirapine exposure (Lakshmi et al., 2010). Anitha et al. (2009) have reported nevirapine resistance among 33-38 per cent of infants infected with HIV when both the infants and their mothers were exposed to a single dose nevirapine.

The findings in present investigations showed that K103N mutations have occurred in 4 mothers and the percentage of nevirapine resistance was less when compared to the previous studies. As nevirapine has a long half-life and drug levels persist for up to three weeks in plasma, it is expected that giving dual NRTI regimens for a period after the women receive single dose nevirapine would suppress viral replication and decrease the risk of developing resistance. For these reasons, the 2009 revised WHO guidelines recommend antiretroviral treatment for all pregnant women with symptoms or CD4 counts < 350 cells/mm³ and a regimen of zidovudine followed by a single dose nevirapine or triple drug prophylaxis starting as early as 14 week, for those with higher CD4 counts (www.who.int). These guidelines are useful and safeguard future treatment options for HIV-infected women and their infants.