Anti-HTLV-1/2 confirmation with INNO-LIA with focus on gp21-alone reactivity

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Abstract

The human t-cell lymphotropic virus (HTLV) is an oncoretrovirus with two variants that causes disease in humans, HTLV type 1 and type 2. The virus have an envelope (env) transmembrane protein called gp21. In a previous study an epitope from the gp21 protein has been proven to react with uninfected serum. The method used to analyze previously negatively screened samples with the HTLV I/II GE80/81 enzyme immunoassay and HTLV I/II Ab Capture ELISA is the line immunoassay INNO-LIA HTLV I/II Score (INNO-LIA). Isolated antibody reactivity with the recombinant protein env gp21 is a common cause of indeterminate results with INNO-LIA. All indeterminate results were collected to see the prevalence of follow up samples and gp21 reactivity. The aim of this study was to evaluate the analytical importance of gp21 reactivity and evaluate if isolated gp21 reactivity could be reported as negative. Among the samples that were analyzed 25 were negative, three were indeterminate with gp21 reactivity and one was invalid. Out of 84 collected indeterminate results there were 55 with follow up samples from the reference laboratory and 29 without. In total there were 61 indeterminate samples, and 23 negative. Among these 84 test there were 62 with gp21 reactivity, of which 6 were gp21 and a second band. The conclusion was that samples with gp21 reactivity when analyzed with the INNO-LIA and interpreted as indeterminate with a follow up sample showing negative or similar result can be reported as negative.

Key words: human t-cell lymphotropic virus, line immunoassay, gp21
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Introduction

Human T-cell Lymphotropic Virus

The human T-cell lymphotropic virus (HTLV) is a retrovirus, these viruses are enveloped, single-stranded, positive-sense ribonucleic acid (RNA) viruses. They have the ability to encode reverse transcriptase enzyme which then converts the retroviral RNA genome into double-stranded deoxyribonucleic acid (DNA). The now linear DNA molecule becomes circular and forms a preintegration complex with help from both viral and host factors. This complex enters the nucleus at random and the DNA integrates into the host chromosome and then replicates with the cell as a provirus. The provirus is thereby replicated and inherited as long as the infected cell lives (1).

HTLV belongs to the subgroup oncoretroviruses. Oncoretroviruses is a subgroup of retroviruses and are highly associated with a variety of cancers; mostly leukemias, lymphomas, and sarcomas. HTLV is a blood borne virus and spreads mainly through blood, intercourse, intravenous drug use and mother-to-child most often by breastfeeding. The transmission is made through cell-associated fluids (1). Which means that the infection can only be spread by cell-cell contact. This comes from the fact that HTLV preferably infect CD4 positive T- lymphocytes (CD4⁺ T-cell) and they require this form of transmission otherwise it is ineffective (2). HTLV is the sole retrovirus that is known to be a direct cause of human cancer. HTLV has shown the ability to cause diseases in humans such as adult T-cell leukemia and lymphoma which is a very rare form of cancer. (3) Two variants of HTLV can cause disease in humans, HTLV-1 and HTLV-2 (1). HTLV-1 is globally spread with a focal point in sub-Saharan Africa, parts of Latin America, the Middle East, southern Japan and Melanesia. HTLV-2 has been shown to be endemic in certain areas such as some American Indian populations and amongst intravenous drug users in urban areas of North America as well as several European countries. HTLV is very rare in Sweden, for example the prevalence of HTLV among blood donors is less than 1/30 000. But it is however more common amongst intravenous drug users in Stockholm. Where it rates at about 2.3% to 3.2% (4, 5).
**HTLV and associated diseases**

The virus is known to have an extremely long latent period which can last for 20-30 years. However this latency does not always occur and in some cases the virus can instead replicate slowly and transform the cells without causing any cytopathic effects. HTLV-1 is known to cause two specific diseases; adult T-cell leukemia/lymphoma (ATLL) and HTLV-associated myelopathy also called Tropical Spastic Paraparesis (HAM/TSP) which is a chronical neurological disorder. Patients that progress to ATLL often show symptoms such as lymphoadenopathy, hepatosplemomegaly as well as skin and bone lesions. The patients that become treated with aggressive chemotherapy often showcase fungal and viral opportunistic infections. The malignant T-cells have a flower-shaped nucleus and are pleomorphic which can be seen in a microscope. HAM/TSP is a disease that causes demyelination in the spinal cord and brain where the motor neurons are the most affected. The disease is believed to be immune mediated and caused by both an autoimmune reaction from the body as well as killing of neurons by the increased amount of cytotoxic T-cells. These patients have symptoms such as gait stiffness/spasticity, low back pain and lower limb weakness. Here the flower-shaped T-cells can be found in the cerebrospinal fluid. It also showcases phoyctic pleocytosis as well as an elevated protein level. In patients infected with HTLV-1 it can also be seen immunosuppression, B-cell chronic lymphocytic leukemia and hematologic malignancies. HTLV-2 has been primarily linked to a variant of hairy cell leukemia caused by T-cells but that is very similar to hairy cell leukemia caused by B-cells. It has also been known to cause the same diseases as HTLV-1 (1). Although the viruses has the ability to cause disease it most often causes asymptomatic infections (6). It is only a small percentage of infected individuals that will develop ATLL or HAM/TSP. The estimation for a person infected with HTLV-1 is that there is a less than 2% risk of developing HAM/TSP. If the person is infected before the age of 20 then there is approximately a 5% lifetime risk of developing ATLL (7). Otherwise the estimated possibility of a patients’ progress to ATLL is approximately 1% to 2.5% (1).
Malignant Transformation

HTLV has the standard retroviral genes, group specific antigen (gag) coding for capsid proteins, polymeras (pol) and envelope (env) genes but also encode the regulatory proteins Tax and Rex for transactivator and regulator of expression. The proteins encoded by the genes are shown in figure 1. The env glycoproteins are gp46 and gp21. Gp46 is a surface glycoprotein whose function is adsorption. The gp21 protein is a transmembrane protein whose function is the fusion of envelope with plasma membrane. The gag capsid proteins are p24 and p19. Both of these capsid protein have a structural function. The receptors for HTLV-1 and –2 has not yet been biochemically identified but they are found in several human and animal cells. The virus is able to infect several different cells and the two HTLV viruses use the same receptor. It has been shown to be the most productive when it infects the T lymphocytes (1). It mainly infects and persists in CD-4 T- cells. The virus genome becomes integrated into the hosts cell DNA (3). When the HTLV-encoded Tax protein binds to HTLV LTR and then increases the transcription of the virus genes. This also leads to the transcription of proto-oncogenes being increased as well. The Tax protein has the ability to enhance the production of the cytokine interleukin 2 (IL-2) and the IL-2 receptor (1). IL-2 also goes by the name T-cell growth factor, it is involved in the control of the clonal expansion of T-cells (8). The increased levels of IL-2 thus leads to the development of uncontrolled growth of the T-cells. This leads to malignant transformation in the cells which causes ATLL (1).
Figure 1. The structure of the human lymphotropic virus-1 (HTLV-1) and the different gene encoded proteins within the virus. The proteins that are of importance for the analysis of HTLV are the envelope proteins, gp46 that is a surface glycoprotein, and especially gp21 that is a transmembrane protein. The group specific antigen proteins of importance is p19 that is a matrix protein, and p24 that is a capsid protein. The nucleocapsid p15 protein, polymeras protein p62/p32, the reverse transcriptase protein, is also demonstrated. The picture is retrieved from the Ohio State Research News website. (The picture is available from: https://researchnews.osu.edu/archive/htlvil2pics.htm)
Diagnostic methods

There exists several different methods that can be used for the analysis and diagnostics of HTLV. The algorithm that was previously used in a majority of Swedish laboratories suggested that there should be a primary and a reference laboratory. The former test algorithm is shown in figure 2. In this it was proposed that the primary laboratory would perform the initial analysis of HTLV which was suggested as an enzyme linked immunosorbent assay (ELISA) test. If this test result would come out as positive then the sample should be tested again twice with the same ELISA assay still at the primary laboratory (9). The ELISA testing that is being used at the primary laboratory is most often in the form of an automated chemiluminescent immunoassay. The most common assay in Swedish laboratories is rHTLV I/II designed for the Architect i2000 (Abbot Laboratories, Abbot Park, Illinois, USA). It is a completely automated instrument that performs all steps of the analysis (10). If the results would come out as negative they would be reported as negative. If the result from that analysis would turn out to be positive the sample would then be sent to the confirmatory laboratory. If two out of three screenings become positive it is sent for confirmation. The secondary laboratory, a reference laboratory, which would be the source of confirmation for the previous test results. There, the samples would first be analyzed with the HTLV I/II GE80/81 enzyme immunoassay (Murex Diagnostics, Dartford, United Kingdom) (Murex) and HTLV I/II Ab Capture ELISA (Ortho Diagnostics, Raritan, USA) (Ortho). These ELISA tests have been proven to be 100 percent sensitive for detection when used in combination with each other. If the sample would turn out negative in both these assays then it would be reported as negative. If the results in these analyses were positive in one or both of the assays it would move on to the next test. The line immunoassay (INNO-LIA) would then be used for the next analysis. The INNO-LIA is a relatively new analysis that is replacing the previous Western blot. Because the INNO-LIA managed to eliminate many of the indeterminate results that the Western blot gave it was deemed better. As well as being easier to interpret results because they give finer bands and is therefore used in its place. If the result was negative it was reported as negative. If the result was positive with a full profile it would be reported as positive. If the result was positive with an incomplete profile or indeterminate there would be a need to send a follow up sample. This follow up sample would then be tested in the last step that is a second
INNO-LIA analysis as well as a polymerase chain reaction (PCR). When the INNO-LIA results are deemed indeterminate there is availability for a PCR analysis to be used for the detection of DNA from the HTLV viruses within the blood. If the test then is negative in either or both of the analysis it is reported negative. If the result is positive it is reported. If it is indeterminate a new sample for retesting is acquired. This algorithm was used as confirmation for HTLV at the Department of Laboratory Medicine at Örebro University Hospital. It was used until the production of one of the ELISA test stopped. Afterwards the same test algorithm has been used with the exception of the first ELISA Murex and Ortho step. Now only the INNO-LIA test is used for confirmation, with a follow up ability with a PCR assay (9).

Figure 2. The previous test algorithm for human T-cell lymphotropic virus diagnostics used at Örebro University Hospital. At the primary laboratory the enzyme linked immunosorbent assay (ELISA) analysis is performed, if the result is negative it is reported. If it is positive an ELISA test is performed twice. If it is negative it is reported. If it is positive or positive/negative it is sent to the reference laboratory. There it is analyzed on the Murex and Ortho ELISA. If it is negative it is reported. If it is positive/negative or positive it is further tested with the line immunoassay (INNO-LIA). If it is negative it is reported. If it is positive it is reported. If it is indeterminate there has to be a follow up sample sent for analysis on the INNO-LIA and a sample for a polymerase chain reaction. If it is negative it is reported. If it is positive it is reported. If it is indeterminate a new test is acquired. Picture created by: Linander H.
**Enzyme-Linked Immunosorbent Assay**

Antibodies are produced against the HTLV proteins, which causes an immunity (1). The most common way of detecting HTLV is by the use of antibodies. There has existed two ELISA methods that when used in combination for HTLV confirmation could result in a completely negative result. Therefore there would be no need for it to be run through the line immunoassay. The methods were HTLV I/II GE80/81 enzyme immunoassay (Murex Diagnostics, Dartford, United Kingdom) (Murex) and HTLV I/II Ab Capture ELISA (Ortho Diagnostics, Raritan, USA) (Ortho). The Murex method had the HTLV-protein rgp21 and a synthetic version of gp46 peptides from both HTLV I and II as antigens. The Ortho method used recombinant envelope and core proteins from HTLV-1 and HTLV-2 as antigens in the coating and detection. The first generations of ELISA’s were first introduced in the middle of the 1980s. When used for HTLV detection it used the whole viral lysate of HTLV-1 as the only antigen. The newer second generation of testing uses recombinant and/or synthetic peptide antigens in combination with or without viral lysate. Another difference is that in the newer method HTLV-2 specific antigens are also being used. This leads to a greater sensitivity for detection of positive specimens (9). These methods belong to the second generation of ELISAs, even though they performed better than the first generation it was still recommended that further testing should be done to safely confirm the results and eliminate false-positive results (11).

**Immunoblot**

Immunoblot or western blotting as it is also called was used before the INNO-LIA came out on the market. The immobilized target is protein, generally it is separated on a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) or isoelectric focusing gel (IEF). The SDS-PAGE separates the proteins according to molecular weight, and IEF separates them according to charge. The sensitivity of the analysis can be increased by using SDS-PAGE and IEF. The proteins are run with pre-decided molecular weight ladders as control for the bands. After the electrophoresis they are transferred with blotting by capillary or electrophoretic transferring onto membranes. Most often the
membrane that is used is made of nitrocellulose. It shows high affinity for protein binding and can easily be treated with detergents. This is done to prevent the primary antibody probe from binding to the membrane by itself before the occurrence of the hybridization. The membrane is then covered with the sample serum. If there is specific antibodies they will bind to the protein antigens (12).

**Line Immunoassay**

INNO-LIA HTLV I/II Score (Fujirebio, Europe N.V, Belgium) is a line immunoassay. Immunoassays are a flexible method for detecting different analytes (12). This method uses specific antigens in the form of recombinant proteins for p19 I/II, p24 I/II and gp21 I/II, and synthetic peptides for gp46-I, gp46-II and p19-I. These are purified and then attached to nylon membranes on the strips (9). The strips are put in trays and are then exposed to a diluent in the form of a buffer and then the test material, in this case, serum is added. If antibodies to the specified antigens are present in the serum these will bind to the strip. A washing solution then rinses of unbound material (11). A conjugate with a goat anti-human IgG covalently labelled with alkaline phosphatase is added and binds to the existing antigen-antibody complex on the strips. Once again unwanted unbound material is washed away with a washing solution (9). By adding a chromogenic substrate solution it generates a color signal that is dark brown if there is an antigen-antibody complex binding (13). Color development is eventually stopped by a stop solution containing sulfuric acid. The intensity of the lines vary due to the amount of specific antibodies that become detected. The interpretation of the test results is a semi quantitative method which helps to moderate the differences when visually read (14).
**The envelope protein gp21**

When performing INNO-LIA analysis there is a possibility of indeterminate results. This is most often caused by a weak (probably unspecific) reactivity or the *env* gp21 transmembrane protein band. The gp21 is regarded as very important when it comes to the diagnostics of HTLV infections. This is due to the fact that the transmembrane protein *env* gp21 antibody reactivity is observed in almost all cases of HTLV-1 or -2 infections and therefore a gp21 reactivity cannot easily be out ruled as negative, which is also why this reactivity deems the INNO-LIA to be interpreted as indeterminate. The *env* proteins have several immunogenic epitopes which causes an antibody response from the infected individuals’ immune system. The epitopes that are of interest are from gp21. The first epitope is GD21-I and the second is BA21, they express amino acids from the HTLV-1 envelope. The recombinant proteins in the analysis are made up of specific portions of the gp21 protein. In a previous study made by the American society of Hematology they tested 17 samples with immunoblot, which were from persons that did not have a HTLV infection. Out of these the GD21-I reacted with none of the samples. But the BA21 reacted with 16 out of 17 uninfected samples. This leads to the conclusion that a certain epitope within the *env* gp21 protein is related to the cause of indeterminate results (15). It has been proven that when it comes to indeterminate results the occurrence of a reactive gp21 band is the most common reason for this. It is not determined if a reaction from an infected individual or an uninfected individual can be differentiated when reactivity in only the recombinant protein for gp21. The problem that these indeterminate results causes is that some of them never gets any other results than indeterminate no matter how many follow up test is made. Therefore it would be a great help if this problem could be eliminated so that it will save time and worry for both the patient and the laboratory (16).

**Aim**

The aim of this study was to evaluate the analytic importance of gp21 reactivity and to evaluate whether isolated gp21 reactivity could be interpreted as a negative test.
Materials and methods

Ethical considerations

An ethical vetting is not needed for this laboratory experiment, since the material that is being used comes from samples that have already been tested for HTLV reactivity by the hospital and they are stored on their premises in a biobank. In this case the previous results were evaluated together with reanalyzes of some of the samples to evaluate the importance of gp21 reactivity. No interference with patient management will be performed because of the results.

Material

The samples used for the performed analysis were serum samples stored at the Department of Laboratory Medicine, Örebro University Hospital. A total of 29 samples that had previously been tested for HTLV I/II antibodies with the Murex and Ortho ELISA methods and resulted in a negative result. These had been frozen at a temperature of -20°C. They were retrieved, thawed and analyzed with the INNO-LIA HTLV I/II kit. All the indeterminate results of previously performed INNO-LIA tests were also retrieved from the database at the Department of Laboratory Medicine, Örebro University Hospital. It was a total of 84 samples. Fifty-five of these 84 indeterminate samples had follow up results at the reference laboratory, leaving 29 samples with none or unknown follow up status. They were then further investigated by contacting the primary laboratory for each test to see if there had been any to the Örebro laboratory unknown follow ups on them.
Method

The INNO-LIA HTLV I/II Score kit (Fujirebio Europe N.V, Belgium) was used to perform the analysis. It was performed according to the manufacturers’ instruction. The experiment starts with the mixing of the wash solution, it should be diluted 5x in distilled water. For each test run there has to be included a positive and negative control, and they always have to be placed in the first two troughs. The strips where placed one per trough, 1 mL sample diluent was added into each trough. The tray was placed on a shaker IKA VIBRAX VXR (IKA-Werke GmBh & Co, Staufen, Germany) and was agitated for a few minutes. Ten µL of the serum sample was added into each trough, and covered with an adhesive plastic film and placed on a shaker to be incubated in room temperature whilst being agitated for 16 hours. The plastic film was removed cautiously to avoid a case of cross-contamination between the troughs. The strips were aspirated and washed with 1 mL of wash solution three times with a five minute incubation in room temperature on a shaker between each round. The liquid was aspirated and 1 mL of the conjugate solution containing goat anti-human IgG labeled with alkaline phosphatase was added and thereafter the troughs are incubated for 30 minutes on a shaker in room temperature. The liquid was aspirated and each test strip was washed an additional three times with 1 mL of wash solution and incubated on a shaker for five minutes between each round. After aspiration of the wash buffer, 1 mL of substrate solution was added into each trough and incubated for 30 minutes on a shaker in room temperature. The liquid was aspirated and 1 mL of the stop solution was added to each trough and were incubated on a shaker in room temperature to agitate for 30 minutes. The stop solution was aspirated and the strips are removed from their troughs with a tweezer. They were then left to dry on an absorbent paper for at least 30 minutes (13).

Interpretation

The strips were placed on a sheet and put into the Epson Perfection V500 Photo (Epson America Inc, USA) scanner. By using the software LIRAS for infectious diseases it was able to scan the picture and then have the results interpreted by the software. The INNO-LIA results was also checked manually, to verify the results from the scanner.
The manual evaluation is done according to the manufacturers’ instructions which is as follows; the lines on the strips are compared to the control lines intensity. All the lines are represented in Figure 3. The background control should become negative otherwise it is a sign of unspecific reactivity within the sample. If the line is absent or less intense than the cutoff line it is negative, 0 (-). If the line is equal to the cutoff line it is 0,5 (+/-). If the intensity is between that of the cutoff line and of the 1+ control line or equal, it is 1 (+). If the intensity is between the 1+ and the 3+ control line, it is 2 (++) . If the intensity is equal to the 3+ control line, it is 3 (+++). If the intensity is greater than the 3+ control line, it is 4 (++++) (13).

Figure 3. The INNO-LIA HTLV I/II Score reference strip. It has all the possible lines exemplified. The control lines are used to determine intensity of the other lines, and the background control is for showing unspecific reactivity. The confirmation lines determine a positive, indeterminate or negative result. The discrimination bands are only taken into consideration if the confirmation lines scored positive. Picture created by: Linander H.
If there are no bands amongst the confirmation lines, the test is negative (Fig.4). If there is one band it is considered negative if it is p19 I/II, p24 I/II or gp46 I/II bands. If it is the gp21 band it is considered to be indeterminate. If there are two bands but gp21 is non-reactive it is considered to be indeterminate. But if there is two bands and gp21 is reactive the test is considered to be positive for HTLV antibodies. If there are three or more bands reactive it is considered positive for HTLV antibodies. The discrimination lines is only to be taken into consideration when the test has proven positive within the confirmation lines. If the bands p19-I and gp46-II are reactive, the result is positive for HTLV-I antibodies. If the band gp46-II is reactive, the result is positive for HTLV-II antibodies. If other combinations occur the results become positive for untypable HTLV antibodies. In the case of an unspecific antibody reactivity it is determined that if there is equal reactivity on all antigen lines in combination with the background control, the result is invalid and needs to be retested. If there is unspecific antibody reactivity on the antigen lines and it is below cut-off level it is considered negative. (17).
Figure 4. A schedule over the interpretation of the line immunoassay analysis results for human T-lymphotropic virus -1 (HTLV-1) and -2 (HTLV-2). The confirmation lines are interpreted first. If there is one band and it is p19, p24 or gp46 it is negative. If it is gp21 it is indeterminate. If there are two bands but gp21 is nonreactive it is indeterminate, but if it is reactive it is positive. If there are three or more bands it is positive. The discrimination lines are interpreted if the confirmation lines are positive. If gp46-I and p19-I is present, the test is positive for HTLV-1. If gp46-II is present, the test is positive for HTLV-2. Picture created by: Linander H.
Results

The INNO-LIA analysis was performed on 29 samples that previously had a negative result when analyzed with the Murex and Ortho ELISA. The controls for each test round were all deemed valid. The positive control shows all the bands for a HTLV-1, and the negative control shows only the control lines (Fig.5). The results are displayed in Table 1. Sample 1 had a gp21 band, the result was indeterminate. Sample 2 to 6 were negative. Sample 7 showed unspecific reactivity and was retested. Sample 8 to 10 were negative. Sample 11 had unspecific reactivity and a streptavidin band it was deemed invalid and had to be retested. Sample 12 had reactivity on band gp46 and gp46-II, the test was negative. Sample 13 to 14 were negative. Sample 15 to 22 is represented in figure 5. Sample 15 had a reactive gp21 band, it was indeterminate. Sample 16 had a reactive p19 band, it was negative. Sample 17 had unspecific reactivity without a streptavidin band and was retested. Sample 18 to 21 were negative. Sample 22 had the gp46 band and the gp46-I, the test was negative. Sample 23 was negative. Sample 24 had the gp46 band and the gp46-1 band, the test was deemed negative. Sample 25 had the p24 band, it was negative. Sample 26 was negative. Sample 27 had a gp21 band, it was indeterminate. Sample 28 to 29 was negative.
Table 1. Results for the samples analyzed with the line immunoassay.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Results</th>
<th>Band</th>
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</thead>
<tbody>
<tr>
<td>Pos control</td>
<td>Pos</td>
<td>All bands for HTLV-1</td>
</tr>
<tr>
<td>Neg control</td>
<td>Neg</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>Indeterminate</td>
<td>gp21</td>
</tr>
<tr>
<td>2</td>
<td>Neg</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
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<td>12</td>
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<td>gp46 and gp46-II</td>
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<td>13</td>
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<tr>
<td>14</td>
<td>Neg</td>
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<td>24</td>
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<td>25</td>
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<td>p24</td>
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<td>26</td>
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<tr>
<td>27</td>
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<td>28</td>
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<td>-</td>
</tr>
<tr>
<td>29</td>
<td>Neg</td>
<td>-</td>
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</table>
Figure 5. Results from the INNO-LIA analysis for samples 15-22 as a demonstration of the outcome. The positive control (Pos C) shows all bands for human lymphotropic virus type 1. The negative control (Neg C) shows only the control lines. Sample 15 shows a faint gp21 band and is indeterminate. Sample 16 shows a strong p19 band, it is negative. Sample 17 shows unspecific reactivity but no streptavidin band. Sample 18 is negative. Sample 19 is negative. Sample 20 is negative. Sample 21 is negative. Sample 22 has the gp46 band and gp46-I, it is negative. Photographer: Linander H
Retesting

Sample 7, 11 and 17 who had unspecific reactivity in the first analysis. The retesting was performed to ensure that the result was because of unspecific antibody reactive and not any other source. The controls were deemed valid. The positive control shows all the bands for a HTLV-1 infection. The negative control shows only the control lines. All of the tests did yet again have an unspecific reaction. Sample 17 and 7 was interpreted as negative because of the lack of a streptavidin band. Sample 11 showed a streptavidin band and was therefore invalid (Fig.6).

Figure 6. Results from the retesting with the INNO-LIA analysis of sample 7, 11 and 17. Retesting was done due to previous unspecific antibody reactivity in the results to secure that nothing had gone wrong during previous testing. Sample 17 and 7 shows unspecific reactivity and no streptavidin band, they are negative. Sample 11 however shows unspecific reactivity and a streptavidin band therefore it is invalid. Photographer: Linander H.
Indeterminate

The 84 samples with previously performed INNO-LIA with an indeterminate result, were divided into two groups; with or without follow up sample sent to Örebro University Hospital (Fig.7). There were 55 samples with follow up tests performed. Thirty-seven of these that came from 17 different patients. With the initial INNO-LIA analyze 23 of these had a gp21 band, three had gp21 and a second band, and 11 had other bands. The remaining 18 of the 55 samples were individual patients that had a negative result on the follow up test. With the initial INNO-LIA analysis they had 15 samples with gp21 reactivity, 1 sample with gp21 and a second band, and 2 with other bands. The 29 remaining samples of the 84 indeterminate samples had no follow up at Örebro University Hospital. Their respective laboratory was contacted. Seven of them had follow up tests performed at their primary laboratory and two of them had a reactive gp21 band in the initial testing. The remaining 5 had shown other bands when analyzed with INNO-LIA. Four follow up samples had been analyzed with rHTLV I/II on the Abbot Architect with a negative result. Two were weakly positive in the follow up screening with the Abbot Architect but were never sent for confirmation. One follow up sample displayed a previous negative result, screened on two ELISAs at the Swedish Institute of Communicable Disease Control. Thus 22 patients/donors with initial indeterminate result had not been subjected to retesting. For 19 of them it could with certainty be said that they had no follow up at all. For three of them there was an identity problem which meant that no information could be found. Of the 19, 16 had had a reactive gp21 band on the initial INNO-LIA. One of these also had a second band. The three samples with no information on follow up samples, showed gp21, gp21 and a second band and other bands respectively.
Figure 7. Results of follow up testing among samples with an indeterminate result when analyzed with a line immunoassay (INNO-LIA). There were a total of 84 samples with an indeterminate result. The ones with known follow up tests at the reference laboratory in Örebro were 55 samples. There were 37 samples that came from 17 patients. Twenty-three of these had gp21, 3 had gp21 and a second band and 11 with other bands. Eighteen of the samples were from individual patients and had negative follow up tests. These had 15 with gp21, 1 with gp21 and a second band, and 2 with other bands. There were 29 samples that were not followed up at the reference laboratory. It was shown that 7 of these had follow up samples at the primary laboratory. These had 2 with gp21 and 5 with other bands. Nineteen of the samples had no follow up tests. These had 15 with gp21, 1 with a gp21 and a second band, and 3 with other bands. Three had no information to be found. These had 1 with a gp21, 1 with gp21 and a second band, and 1 with other bands. Graphic created by: Linander H.
Discussion

The results of the 29 samples that had previously been tested with the Murex and Ortho ELISA with a negative result and now were analyzed on the INNO-LIA were all negative except for four samples. Amongst these four samples 3 were indeterminate and one invalid. There were 25 tests that were negative. The one test that were determined to be invalid was because of unspecific reactivity and reactivity in the background control. All the test that had unspecific reactivity were retested to confirm that the reaction was because of the sample and not because of a source of error in any analysis step. An example of a source of error is the human factor since the analysis performed on the samples were done manually. They showed the exact same result as previously and could therefore get a final result. The three test that became indeterminate all had one gp21 band and no other bands. This fact makes it indeterminate because of the importance of the gp21 band, and the uncertainty surrounding it. These samples that were reanalyzed all previously had a negative result. In the reanalyze none of them became positive which is very good because it shows that the previous test algorithm worked and no positive test had slipped through. There were only 3 samples among the 29 samples that had previously been negative with the ELISAs that became indeterminate. Meaning that the loss of the Murex and Ortho ELISA has not had a great impact in the difference of prevalence of indeterminate results.

The INNO-LIA samples with or without known of follow up were a total of 84 indeterminate samples. There was 55 with known of follow up, 37 of these were from 17 patients, and the remaining 18 were from 18 individuals. Considering the samples that had been tested with the INNO-LIA and became indeterminate without known of follow up test, there was a total of 29 samples. Of these 7 had follow up tests at the home laboratory, 19 did not and the remaining three had no information. There could be no information of these three because they had been identified with a blood donor number that could not be traced in retrospect. The recommendation from the reference laboratory is that when a HTLV test becomes indeterminate a follow up sample should be obtained and sent together with a whole blood sample to the reference laboratory for further analysis using serology and PCR.
The seven samples that actually had follow up samples were only analyzed at their “home laboratory”. Since they were not done in the reference laboratory none of them had thus been performed on the INNO-LIA. Four had been analyzed on the Architect instrument only, with a negative result and was thus regarded as negative with unspecific reactivity in the first screening test analyzed. Similarly one of the tests had previously been tested with two ELISAs at a different laboratory and became negative. However two samples had follow up tests that were weakly positive. For unknown reasons they were not sent to the reference laboratory. Altogether 5 out of 7 of these initially reactive samples could be confirmed negative in the second analysis. Since an indeterminate result cannot be said to be either positive or negative it is of great importance that the samples have follow up tests performed. The information about sending a follow up test is always included with the result. So the question is then why does the clinic in charge of the patient disregard the instructions given to them about the test results from the reference laboratory. This is a definite problem, and might need to be more looked into too see how the reference laboratory could get the information out of the importance of following-up indeterminate results. Maybe some information campaign from the reference laboratory would be an adequate action.

When investigating the occurrence of gp21 in all the indeterminate samples, this is what was found. The 37 samples with follow up had when analyzed with the INNO-LIA 23 samples with gp21 reactivity, 3 samples with gp21 and a second band, and 11 with other bands. The 18 samples with follow up had 15 samples with gp21 reactivity, 1 with gp21 and a second band, and 2 with other bands. The 7 samples with follow up at its home laboratory had 2 samples with gp21 reactivity and 5 without. The 19 samples without any follow up had 15 samples with gp21 reactivity, 1 with gp21 and a second band, and 3 with other bands. The 3 samples with no information had 1 with gp21 reactivity, 1 with gp21 and a second band, and 1 with other bands. This shows that out of 84 samples there were 62 that had gp21 reactivity, 6 of which were with gp21 and a second band. Showing the fact that the majority of samples with an indeterminate result are because of the gp21 band. In total there are 61 still indeterminate samples and 23 negative.
Solution

A strategy that feels fully relevant and safe to tackle the problem with indeterminate reactions in the INNO-LIAs would be to perform a follow up test together with the PCR. If the result then is negative or still indeterminate and with a negative PCR the patient should be considered as not infected. If any of the test turn positive more analyses are to be performed to validate this result. Importantly no test with positive reactions in follow up analysis was found in this study. When using both the INNO-LIA and PCR you get to both check for antibodies and virus DNA. Hence when there is indeterminate antibody results you can then use the PCR and when not finding virus DNA be certain of negativity. Thereby eliminating the risk of letting a positive test slip through. It is important to both use the INNO-LIA and the PCR to definitely ensure that there is no infection before reporting it as negative as especially HTLV-2 infections is associated with both low levels of antibodies and viral DNA. There are several problems caused by the indeterminate results. First, the persons getting this result are usually healthy donors or under investigation for In vitro Fertilization. A reactive result is for these individuals traumatic and can cause delay and cost in the health care system. Secondly, as the test material is serum for the INNO-LIA, and whole blood for the PCR the patient often have to be called again to retrieve this sample. Based on this study (no Indeterminate results could be verified as positive in the follow up sample) an even more rapid approach could be considered, i.e. that indeterminate results on low risk patients (i.e. screening samples with no association to high endemic settings or iv drug users) could be interpreted as negative. This approach has to be further evaluated but seems based on this study as an equally safe approach.

However this approach would be limited to low endemic countries were the risk groups of infection (mainly intravenous drug users in an urban environment) can be identified. Thus this is not recommended for other countries because of the difference in prevalence of HTLV infections around the world. If a follow up sample is obtained the sample should be taken several weeks after the first test was taken. This to ensure that the gp21 reactivity is not a sign of an early developing infection. It is also important to contemplate the occurrence of infections from other strains of HTLV, it is very rare but
has been proven that there are cases where people have been infected by HTLV-3 and -4. They are often asymptomatic and has shown a prevalence in Central Africa (18). A thorough patient anamnesis can in these cases be of great help. Though this occurring would be an immense rarity in Sweden. As previously mentioned the gp21 band is detected in almost all cases of both HTLV-1 and -2 infections (15). That is why there is much uncertainty around it and why reactivity in only the gp21 band is deemed indeterminate. When taking into considerations the results gathered from this study, tests that had previously been determined as negative with the Murex and Ortho ELISA showed 3 indeterminate gp21 samples. In three of the test that were indeterminate and had follow up tests they were analyzed with PCR and became negative, two of these had the gp21 band. It has been proven in previous studies that in recombinant proteins from the gp21 protein there are different epitopes. One of these epitopes, BA21, were determined to be a direct source of unspecific antibody reactivity. It was a direct cause of indeterminate results when tested on what was already known as HTLV negative samples (15). With all of these factors being taken into consideration the results can with certainty be reported as negative when a follow up sample for a gp21 reactive test is tested with both the INNO-LIA and the PCR.

**Conclusion**

Samples with a gp21 band reactivity only analyzed using INNO-LIA and interpreted as indeterminate with a follow up sample showing negative screening test or similar result can be reported as negative. The risk that it would actually turn out to be positive at a later stage is so slim that the result can be deemed negative. The strategy might be of relevance also in countries with higher prevalence of the disease but larger studies in these areas has to evaluate this.
References


