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RESEARCH ARTICLE

Title: Surveillance of HTLV-l/ll and their dominant genotypes among healthy blood donors in Karachi, Pakistan.

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Abstract

A Cardinal ambition behind this study is to appraise the ubiquity of HTLV-l and HTLV-ll among blood donors, because some life threatening diseases or viruses are spreading by blood including HTLV-l and ll. HTLV-l is the first discovered human retrovirus isolated in1979 from a vitro cultured cell medium using T-cell to a victim suffering from skin lymphoma of T-cell. HTLV-l/ll allied with malignancy of T-cell, a rare cancer of the immune system's own T-cells known as Adult T-cell lymphoma/leukemia (ATL), some neurologic disorders (HAM), Tropical spastic parapasis and other Oncogenic infection. HTLV-ll is also a closely homology virus of HTLV-l but not linked to lymphoproliferative disorders. Transmission routes are sexual contact, blood and blood component, syringes, from mother and by breast milk. Terrestrial spreading occurs far-off twenty-five years and the high ratio found in Japan, America, Africa and Iran. In many part of the world different diagnostic test are performed to screened the blood and its product to HIV, HCV, HBs-ag and HTLV-l/ll which obviously played an important role in decreasing of virus transmission. Unfortunately in developing countries including Pakistan HTLV-l/ll could not get attention as other viral infection, Proposed study is the first detailed study from Pakistan which aim to evaluate the surveillance for HTLV type l and ll in healthy blood donors. In this project we analyzed 100 healthy blood donor's serum sample by RT-PCR and found negative all of our samples while all the calibrators and control were satisfactory. But HTLV-l/ll virus could not be negligible because we analyzed a small sample size (n=100). We hope that Health authorities will take immediate step on this virus surveillance data collection in Pakistan as soon as possible, because of presence of this virus in our next door neighboring country Iran in a noticeable ratio.

Introduction

It is the first exogenous horizontally transmitted retrovirus which caused certain hematologic and neurologic disorders in human **[1].** Initial discovery take place in Japan in 1977 which indicate the association to CD4 + lymphoproliferative T-cell leukemia or lymphoma termed as ATLL indicated by the clonal integration of HTLV-I provirus in to the tumor cells **[2].**Type -II human Retrovirus is a virus closely related to type-I (Human T-cell lymphotropic virus), which go halves around 70% genomic similitude (structural resemblance) by means of Human Retrovirus type-I**[3].**The identification of HTLV-ll was carried out in1882 as of a patient suffering from hairy cell leukemia this virus intended for CD8+ T cell malignancy[**4**]. HTLV-I infection may influence by HLA subtype and a neurologic syndrome documented as HTLV-I-associated myelopathy also called tropical spastic paraparesis

[HAM/TSP]**[5,6]**.Some other abnormalities also have been reported like arthropathy, polymyolities, bronchiolitis, uveitis and elevated calcium level**[7].** Flower like or multiplies of cell nuclei observed in peripheral blood film **[8]** .Transmission routes are same as that of hepatitis B, C virus and HIV from blood and related products, by critical items specially needle sharing, sexual practice, to fetus from mother and by breast milk also **[9].**

Physio pathology:

Majority of people got infection of HTLV may not develop any disease related it.Among infected individuals the few, about 5%, will show the symptoms and complications, in chronic stage [**10**].The two important diseases originate by HTLV-I are Adult T-cell leukemia/lymphoma (ATLL) a form of blood cancer, HTLV-I-associated myelopathy (HAM) or tropical spastic paraparesis (TSP)**[11].** An excitation or swelling of nerves of spinal cord that creates inflexibility or loss of strength of legs as well as backache, weakness of bladder and constipation also occur. Other symptoms and complication like inflammation of the eye (uveitis), joints (arthritis), muscles (myositis), lung (alveolitis) and skin (dermatitis) are seen. These conditions are less common ATLL than HAM/TSP [**12**]. It is not so apparent what diseases HTLV-II causes, but there is a little indication that HTLV-ll infection is linked with neurological rather than blood disorders. These diseases are caused by intracellular proviruses that cross by the formation of a "virological synapse", and the viral genom move onward from one to another cell then further step is replication [**13**]. The virus affects T-lymphocyte and the Proliferation of affected T lymphocytes increased. CD4 lymphocytes mainly affects by HTLV-1, while CD8 lymphocytes affected by HTLV-ll. HTLV-1 also have the capability to infect other cell types, because of the diverse pathogenesis of HTLV-1. Research shows that a universal glucose transporter (GLUT-1), exploit as a receptor for HTLV-l. The genes of HTLV-1, in the pX region, a transcriptional activator Tax encoded is perceiving to perform a valuable role in inflammation, immortalization, and oncogenesis by means of its pleiotropic action [**14**]. The patients suffering from HAM/TSP, a number of cytokines, chemokines and matrix metalloproteinase such as tumor necrosis factor-α (TNF-α) transactivated by Tax protein. A report from Japan also indicated that HTLV-1 tax mRNA expression was higher in HAM/TSP.The study of fresh leukemic cells isolated from ATL patients, shows about 60% of cases do not express the tax transcript[**15**] . These results propose that Tax is important for malignant transformation but not crucial for the upkeep of leukemic cells *in vivo*. Recently, a different basic leucine zipper protein encrypt by the corresponding strand of the HTLV-1 genome, having as a name HTLV-1 basic leucine zipper factor (HBZ), was characterized . It exibit in entire ATL cells, boost reproduction of T-lymphocytes in its RNA form , conceal Tax-mediated transactivation through the 5' LTR , assist CD4+ T-lymphocyte proliferation shows in transgenic mice , and exaggerate infectivity and persistence in HTLV-1-inoculated in rabbits[**16**] .Right away these research help us to understand the pathogenesis of this virus .After the infection the host proceed an immune response which is antigen-specific against the HTLV-1 antigen. Cytotoxic T-lymphocytes of the host's immune system respond by releasing cytokines and fight against infection. Promote across the endothelial migration of lymphocytes above the blood-brain barrier. Cytokines are within the CNS, demyelization is brought by observer cell injury. This stage is persistence, slowly proceed, and elicit only symptoms 20–30 years after infection.

The approach of virally stimulated tumors:

These mechanisms can be classified into acutely or slowly transforming respectively. The viral particles bear a gene that codes for an overactive oncogenes termed viral-oncogenes (v-onc) in acutely transforming viruses[**17**] , after expression v-onc the contaminated cell is altered and in slowly transforming viruses, viral genome insertion take place which is an mandatory part of retroviruses, in the host genome close to a proto-oncogene. Other transcription regulation fundamentals like viral promoter in turn cause over expression of that proto-oncogene, which induces proliferation of cells in an unrestrained manner [**18**].

Tax gene:

Tax is a 351-amino acid, 40-kDa protein (38) which seems to be responsible for the pleiotropic effects that an HTLV-1 infection can have on susceptible cells [**19**]. This phosphoprotein functions as a transcriptional activator of the viral genes by recruiting transcriptional co activators to the long terminal repeats flanking the HTLV-1 genome Structurally, Tax contains both a nuclear localization and nuclear export signal, numerous leucine zipper-like sequences, a transactivation domain, and other DNA-binding sites (see the figure below)**[20**].The transactivation domain is known to regulate nuclear factor-kappa B (NF-κB) . In the cell, Tax is mainly localized in nuclear and perinuclear speckles, and it also co localizes with centrosomes. Expression of Tax alone is sufficient to induce detrimental changes in normal T-cells, especially centrosomes over amplification [**21]**, aneuploidy, and chromosomal instability.

Epidemiology:

As for as the epidemiology is concern the highest incidence rate was found in specific areas in Southern Japan (specially in Kyushu, Okinawa and Shikoku), in Caribbean including Trinidad, Jamaica, Martinique, Haiti, Barbados ,South America, Southern United State, Africa Philistine, Northeastern Iran ,Middle East and among some Indian tribes [**22**]. Approx 15-20 million people worldwide affected from HTLV-l. The major molecular subtypes of HTLV-l and their incidence are: subtype (A) also named Cosmopolitan subtype that is endemic in Japan. Subtypes B , D & F in Central Africa while the endemicity of C subtype is in Melanesia and subtype E in South and Central Africa[**23].**The reliable research studies concern to epidemiology have based upon detection of antibodies to HTLV-I in serum. Techniques mainly used for this purposes are (EIA), which conformed by another method such as, immunofluorescence (IFA) or ELISA radio-immunoprecipitation (RIPA), Western blotting and Polymerase chain reaction (PCR) assays by detecting proviral DNA of HTLV-l. Proviral DNA sequencing or restriction fragment length polymorphism (RFLP) analysis, are also used for viral sub typing **[24, 25].**HTLV is related to PTLVs(primate T lymphotropic viruses).Infection or diseases caused by the members belonging to this family are known as Human T-lymphotropic viruses, and others that infect old-world primates are belong to Simian T-lymphotropic viruses. HTLV-ll is another type of HTLVs that are also associated with human diseases commonly known as hairy cell leukemia. A remarkable epidemiology of HTLV-l because of its restricted geographic seroprevalence is found to be important characteristics. A low prevalence and sporadic cases of HTLV-I has been reported in many parts of the world China, Taiwan, Korea, Iraq, India, Kuwait and the previous Republics of the Soviets Union. in Caribbean island Seroprevalence of HTLV-I infection differ from 3-6% to 6-3%[**27**] .The highest geographic prevalence of HTLV-I infection In the world is The southwest district of Japan is found to be 1.2 million people have antibodies against HTLV-I it is the highest geographic prevalence. HTLV-I seroprevalence increases also with age and it is high between subjects above 40 years of age[**28**].Increased seroprevalence of about repeated infection has been seen in females, because during sexually active years the virus transmission is more likely efficient to females from males. The rate of HTLV-I infection is greater in family members as compare to general population. So it is suggested that shared environment and repeated close contact could be important in the transmission of this virus. In Israel the sporadic cases of ATL seen among immigrants Jewish originated from Mashhad further research determined the high risk of HTLV-I infection in this group was about 12%. It might be due to high rate of interfamilial marriages among its members as well as ethnically segregated population .In a similar, study among Mashhad born 23% of seropositivity and existence of cases of spastic paraparesis was reported .

Transmission:

There are three important routes of transmission of HTLV-l are commencing to infant by feeding mother breast milk. it is one the main cause of HTLV-I infection [**30**]. In Japan Studies showed that the increased occurrence ratio of HTLV-I infection in carrier mother's children of was (21%) as compare to children like 1% in the general population .Approx 85% mothers who were infected by HTLV-l transmit virus to their children. The breast-feeding duration also influence the risk of spreading. However, the provirus in the circulation of cord blood is derived from migrated maternal cells which are not a part of the baby blood distribution .So; transmission of intrauterine could not be a main lane [**31**]. Sexual contact is the 2nd important way of HTLV-I transmission. About 60% Transmission occur from man to woman that is more frequent than transmission occur by woman to man which is 0.4%. Like other retroviruses such as HIV, It can be transmitted through homosexual activity. The third rout is blood transfusion. In donors blood lymphocytes the proviral DNA acts as an infectious agent. The probability of sero-conversion is about 44% in a contaminated blood recipient [**33**] .Thus, an efficient blood screening system is essential for in endemic areas to minimize the HTLV-I transmission. The whole blood and its components, such as packed red blood cells and platelets are also the important sources. Fresh frozen plasma, are not to be consider as a source of virus transmission. The reservoirs of HTLV-I are white blood cells. After one week storage of infected blood the transmission probability of HTLV-I would be decrease .It can also be transmitted by drug addicts during needle sharing[34].

Symptoms and diseases:

This virus has been connecting in a number of diseases commonly include myelopathy and adult T-cell leukemia/lymphoma,HTLV myelopathy: such as convulsive changing in muscle function also with motor and sensory alteration or other lower limbs weakness. Changing in neurological conditions, mostly cause upper motor neuron lesions involving descending motor pathways, accompanied by of hyper excitability also observed named as 'Clonus' , bladder dysfunction leading to bladder cancer. Sexual disorders such as Erectile dysfunction (ED) and Temperate mental impairment also seen [**35**].Adult T-cell leukemia is also an important disorders caused by this

virus .There are four types of clinical disorders are found regarding to ATL including : symptoms comprises mild and intrusive clinical course called acute stage , hypercalcemia (increased level of calcium), bone diseases (malignant and benign), lungs diseases, and cancer in which increase lymphocyte count , enlargement of liver and the spleen or Hepatosplenomegaly[**36**].Dermatological scratch including indolent, indurate ,nodular, , erythrodermal or exfoliative, second type is submit as Smoldering Acute T-cell lymphoma which shows symptoms like atypical lymphocytes of 5% or less Malignant cells with monoclonal proviral integration, skin lesion, Pulmonary involvement (occasional) lymphadenopathy or other visceral involvement, possible elevation of the serum lactase dehydrogenase level.

Materials and methods:

Criteria followed for donor selection:

There are 95(95%) male and 5(5%) female donors; ages between 20 to 45 years were enrolled. Givers accomplish the criteria for blood donation, incorporated a discussion to trace the history of earlier blood transfusion ,suffering from infectious or heart diseases, type of surgery and foreign traveling details followed by complete blood count(CBC) and Screening to (HIV,HBs-ag,HCV,VDRL,Malarial parasites).After the confirmation of negative screening samples were collected.

Sample collection:

1000 healthy blood donor persons were registered in this study. Blood sample were 2.5cc collected from all individuals in tubes containing, EDTA anticoagulant for Complete Blood Count (CBC) to analyze total WBCs count, total RBCs count, hemoglobin, MCV , MCH and 5cc collected for screening and molecular analysis, in plane tubes (no anticoagulant).If needed then stored in -20^0c

DNA Extraction: The RNA/DNA was extracted from serum samples by DNA/RNA extraction Kit (Viral Gene-spin $^{TM}{}_{viral}$ DNA/RNA Extraction Kit).

Procedure: In a sterilized eppendorf take 150ul of samples. Add 250ul of lysis buffer then vortex for 15 sec.Incubate at RT (15-20^0c).Add 350ul 0f binding Buffer and vortex 15sec.Transfer the sample inoculums. Centrifuge (13000rpm/min).Add 500ul of washing Buffer A. Centrifuge (13000rpm/min).Add 500ul of washing Buffer B.Centrifuge (13000rpm/min).Prepare the column to 1.5 ml tube. Add 30-60ul of Elution Buffer and incubate for 1min, again centrifuge (13000rpm/min).Finally discard Spin column.

RT-PCR:

Primers: *tax* (forward, GGA TAC CCA GTC TAC GTG TTT G) 56.14 nMol converted to the stock of 100uM/L concentration that is 561.4 uM/l.and reverse, CGG AAC ATT GGT GAG GAA GGC) 67.24 nMol or 672.4uM/l according to GenBank accession no EO3562. MT-2 cell line DNA was used as positive control. Then prepare working solution (10ul stock solution+90ul TE Buffer) Prepare Tris Buffer (2ml EDTA+10ml HCL) for Lypholysed Primer.

By using pure Taq Ready-To-Go PCR Beads which contains buffer, dNTPs, recombinant pure Taq DNA polymerase enzyme, stabilizers and BSA. All which have been pre treated to minimize the contamination and handling error, only Template DNA and template specific primers need to be added. For each reaction, add the following to a tube containing a PCR bead: and run by CFX96 Touch Deep Well™ Real-Time Instrument

Master Mix:

5′ (forward) primer (25pmol)	2.5ul
3/(reverse)/backward)primer(25pmol)	2.5ul
Template DNA	07ul

Sterile high quality water	13ul
Final volume	25ul

Amplification Protocol:

Standard Curve 50 Cycles	Step	Time	Temp
	Reverse Transcription	10 mins	55 oC
	Enzyme activation	2 mins	95 oC
	Denaturation	10 secs	95 oC
	Data collection *	60 secs	60 oC

Fluorogenic data collected during this step through the SYBER green channels

Results & Discussion:

For the evaluation of HTLV-l and ll we investigated 100 healthy blood donors by RT-PCR technique using serum samples. Primers for this process were precise for the tax region and pol common to HTLV-I and HTLV-II,**.** According to our results all the samples were showed absence of targeted genomic sequences for HTLV-I and HTLV-II.While.The results of calibrator and controls were satisfactory.Now a days there are different type of diseases including Hematologic and Neurologic are seems to be in increasing numbers. Unluckily there is no defined treatment for patients suffering from these types of unknown infections and the last choice for treatment purposes live saving drugs (cortisones)are used which also create other complications. HTLV-1/ll, is one of those initial human retroviruses discovered and caused T-cell malignancy like ATLL,and Neurologic diseases .Unluckily the lack of exact information of sero prevalence rates in dissimilar population specially in Pakistan can commence threats regarding to HTLV-l/ll .So for the prophylactic measures it should be reduce the rates of viral transmission from infected individuals. The elevated percentage of uncertained result of the lab test for Human Retro RNA virus comprising a relationship for lymphatic tissue infection has been a wide-reaching challenge Thus blood donors lab examination regarding these viruses should be necessary in every blood banks because blood is the important source for the transmission of blood born diseases and According to the International Retro Virology Association more than 95% of people infected with HTLV-l and ll have little or no symptoms at all. Some treatments are available for diseases associated with HTLV, such as HAM and ALL. No vaccines are available to prevent against HTLV-l and ll .Many countries in the world take steps and they screened the blood donors to find out the HTLV-l/ll. In Pakistan it is very important to evaluate the ratio of HTLV-l in our Population because the world wide study showed higher epidemic ratio in the Jamaica that is (2.1%), in United State (0.004%), Brazil (0.42%) and France (0.004%).The neighboring countries of Pakistan like in Iran (Mashad) 0.77%. HTLV-1 seroprevalence rate among blood donors are found. in Khorasan (Iran) there is an increased risk of cancer due to this virus. Infection is highly prevalent among HIV infected IDU patient such as 16.33% in Ahwaz (Iran**).** Unfortunately from Pakistan there was no detailed study found dealing with the prevalence of HTLV-l/ll, even simple antibody screening tests like rapid immunochromatography and ELISA are not available in the clinical/pathological diagnostic Laboratories of the

country. It is also a fact that the authenticity based conclusion making in health requires the accessibility of sound data. We here at Jinnah University designed this study to assimilate and disperse knowledge regarding this disease in our communities in Karachi

For this purpose we selected 100 serum samples of healthy individuals blood donors (normal CBC parameters and screened for HIV, HCV, HBs-ag, VDRL,malarial parasites) from Blood Bank. Then The suspected RNA/DNA was extracted from serum samples by DNA/RNA extraction Kit (Viral Gene- spin $^{TM}_{viral}$ DNA/RNA Extraction Kit).Amplified the samples using Primers: *tax* (forward, GGA TAC CCA GTC TAC GTG TTT G)56.14 nm converted to the stock of 100uM/L concentration and reverse, CGG AAC ATT GGT GAG GAA GGC) according to GenBank accession no EO3562.By puRe Taq Ready-To-Go PCR Beads (Amersham Biosciences,UK) which contains buffer,dNTPs,recombinant pure Taq DNA polymerase enzyme, stabilizers and BSA.Using RT-PCR Method because this technique has debatably been the most noteworthy development in the past several decades with regard to the ability to differentiate and match up to the genomes of viruses. Within 50 cycles by RT-PCR Instrument(Thermal cycler) time intervals were taken 3 and 1/2 hours. Our all samples showed negative results. Negative RT-PCR results point out the nonexistence of equally human T-cell lymphotropic virus types I and II. A negative test outcome does not leave out the option of exposure to human T-cell lymphotropic virus types I and II (HTLV-I or HTLV-II). May be the reason behind the negative results is that Tax gene is not expressed at continuously higher levels in carriers and HAM/TSP. Also the amplified sequence was a small fragment of Tax and it is fragile protein and most mutations even small changes inactivate the activity of tax. This project was applied to a random population of asymptomatic carriers that's why we were not able to detect any positive specimen. May be if we look for the same virus in different clusters of subjects like patients with liver disorders or immunocompromised patients then may be a different outcome will appear. So, it is suggested that we may go forward with this base line data and procedure in future to start for a large data study.

Conclusion:

Avoidable contagions become a foremost source for death and lack of sufficient control, and physical strength or mental ability in socio-economically disadvantaged areas of world, and also in Pakistan. Contrasting other infections HTLV-l/ll could not get attention. We believe that the current study is the first study on HTLV-l/ll surveillance from Pakistan. We used a limited group of individual's i-e. Blood donors but it is suggested to include other cluster also to rule out the exact magnitude of this virus in our communities. We also like to mention here that we made an effort to take this deadly infection on board and to establish awareness among our civilians and researchers regarding these viruses and their associated infections and suggest that a comprehensive and detailed study should be carried out to uncover the exact surveillance of this virus in Pakistan.

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