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Evaluation of Multiplex Polymerase Chain Reaction (PCR) as Prognostic Tools for Chronic Myeloid Leukemia (CML) Patients in Resource-Limited Setting at Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia.

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This is to certify that the thesis prepared by Saifu Hailu Chala entitled "Evaluation of multiplex polymerase chain reaction (PCR) as prognostic tools for chronic myeloid leukemia (CML) patients in resource-limited setting at Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia" and submitted in partial fulfillment of the requirements for the Master's degree in Clinical Laboratory Sciences (Hematology and immunohematology specialty track) complies with the regulations of the university and meets the accepted standard concerning originality and quality.

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Abbreviations

ABL-Abelson

ALL-Acute Lymphoid Leukemia

AML-Acute Myeloid Leukemia

BCR-Breakpoint Cluster Region

BM-Bone marrow

CCR-Complete Cytogenetic Response

cDNA- Complementary deoxyribonucleic acid

CHR-Complete Hematologic Response

CI – Confidence interval

CML-Chronic Myeloid Leukemia

CMR-Complete Molecular Response

DMR- Deep molecular response

EMR-Early Molecular response

EUTOS-European Treatment and Outcome Study

FISH -Fluorescent *Insitu* Hybridization

FNF- False-negative fractions (1-sensitivity)

FPFFalse-positive fraction (1-specificity)

GIPAP-Gleevec International Patient Assistance Program

GTC-Guanine thiocyanate

HSM-Hepatosplenomegaly

HU-Hydroxyurea

IRSIS-International Randomized Study of Interferon and STI571

IS-International Scale

M-Major

m-Minor

MMR- Major molecular response

MR- Molecular response

MRD-Minimal residual disease

NCCN-National Comprehensive Cancer Network

NPV- Negative predicted value

OD-Optical density

PB-Peripheral blood,

PBS-Phosphate buffer saline

PCR-Polymerase Chain Reaction

Ph-Philadelphia

PPV-Positive predicted value

Q-rt-PCR-Quantitative reverse transcriptase Polymerase Chain Reaction

RCLB-Red cell lysing buffer

qRT- PCR-Real Time-Quantitative Polymerase Chain Reaction

TASH-TikurAnbessa Specialized Hospital

TKI-Tyrosine Kinase Inhibitor

TNF-True negative fraction (specificity)

TPF- true positive fractions (sensitivity

WBC-White Blood Count

x^2- chi-square

Abstract

Background:-The hallmark diagnostic tool for Chronic myeloid leukemia (CML) is Philadelphia chromosome t (9; 22) (q34; q11)) which gives rise to bcr-abl1 fusion oncogenic protein. Qrt-PCR of bcr-abl1has been established as a prognostic tool since the introduction of TKI drugs. But, in a resource-limited setting, like Ethiopia, multiplex reverse transcriptase PCR, which is used to be as a screening tool, could be adopted into the clinic as a relative prognostic tool than relying on hematological response.

Objective: - to evaluate multiplex polymerase chain reaction (PCR) as prognostic tools for chronic myeloid leukemia (CML) patients in resource-limited settings at Tikur Anbessa Specialized Hospital (TASH).

Method: - Hospital-based Cross-sectional study design was used for a total of 114 confirmed CML patients who were enrolled at the Hematology Clinic of TASH. Multiplex reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative reverse transcriptase PCR were performed at AHRI molecular lab, and Leipzig hematology clinic, respectively. The descriptive statistic and ROC curve using excel were used to assess the demographic feature of the patients and to evaluate the prognostic value, respectively, and thereby the most valuable cut-off multiplex PCR was calculated in comparison with QRT- PCR.

Result: - of the total enrolled study subjects (114), most of the transcripts were major fusion gene (M-bcr-abl1), b2a2 (28.1%), b3a2 (49.1%), and co-expressed were b2a2/b3a2 (0.9%). All new cases and follow-up patients who were positive with the multiplex RT-PCR method for bcr-abl transcript were more than 95% agreement with quantitative PCR compared to the limit of detection sated for multiplex PCR. Multiplex RT- PCR at a cut-off 0.31% IS and above, in comparison to the standard prognostic tools (Qrt PCR) has a comparable result; sensitivity 96.6% and specificity 88% at the area under the curve 0.977(97.7 %) and CI 95% (0.952 to 1, P-value 0.0001).

Conclusion: - Follow-up of patients on the hematological parameter is rather crude and not sensitive enough to modify the treatment regimen. But, the use of multiplex PCR, besides as screening tools, can provide an early sign of relapse as low as 0.31% IS bcr-abl transcript. As compared to Qrt-PCR, it is relatively cheap and accessible to be adopted into the routine clinic.

Keyword: - BCR-ABL- RT-PCR, chronic myeloid leukemia, molecular response, Ethiopia.

1. Introduction

1.1 Background

The first hematological malignancy to be related to a specific chromosome abnormality was chronic myelogenous leukemia (CML). It happened due to reciprocal translocation between 9 and 22 chromosomes which was identified by Nowel and Hungerford in 1960 called the Philadelphia chromosome(1). Per year there were around 1.8 new cases diagnosed for CML from 100,000 individuals, with an average age of 65 years. Of all adult cases of leukemia 10% to 15% account for CML(2). Greater than 90% of all CML and 10 -25% of ALL are associated with a translocation between chromosome 9 and 22chromosome(3). It is characterized by three phases when left untreated: 1) a chronic phase of four to five years manifest by myeloid hyperplasia with the domination of mature granulocyte; 2) an accelerated phase with a short time of duration at which myeloid element lose their capability to differentiate; and 3) an inevitably, a blast phase of acute leukemia of myeloid(70%) or lymphoid(30%) phenotype(4).

The molecular hallmark and causative agent of chronic myeloid leukemia is the fusion v-abl (Abelson) murine leukemia viral oncogene homolog 1 (ABL1) gene on chromosome 9 and the breakpoint cluster region (*BCR*) gene. More than 95% of CML shows the transcript e14a2 (b3a2) or e13a2 (b2a2), such as major BCR-ABL1coding for a p210 protein, while minor like 1% to 2% of CMLs e1a2 show coding for p190 protein(5). Also other rare transcripts, like e19a2, e13a3, e14a3, e1a3, e6a2, e2a2, and e8a2 are rare and usually anecdotally reported(6). The main cause of the resulting *BCR-ABL1* fusion protein is a constitutively active kinase and can drive uncontrolled proliferation in myeloid precursor cells(2).

The Philadelphia chromosome was mainly detected in the cytological study, but BCR-ABL1 can be detected using qualitative or quantitative polymerase chain reaction (PCR).In the current situation quantitative PCR (qPCR) as reported in the International Scale (IS) (qPCR) is a standard criterion for various international treatment guidelines(7).

Quantitative*BCR-ABL1* monitoring is currently being used to know if the optimal response is achieved promptly. In cases of CML, outcome predictions, as well as the risk of progression to accelerated phase and blast crisis, have been associated with molecular response kinetics(8). Screening of specific recurrent genetic abnormalities is very important for disease

evaluation, optimal risk stratification, and treatment planning, which is recommended by the USA National Comprehensive Cancer Network(9).

To amplify specific DNA sequences PCR is the central technique used for molecular testing. The amplified DNA sequences can be sequenced, sized, or labeled with probes to identify alterations in the DNA, including base-pair mutations, insertions, or deletions. PCR is mainly divided into 3 reactions, each usually assumed to occur over time at each of three temperatures, namely: denaturation, annealing, and polymerase extension that results in the exponential amplification of a targeted DNA sequence between two targeted DNA primer regions. This results in a large number of double-stranded templates available at the end of the cycles and favor re-annealing with itself at the expense of efficient primer binding and extension(10).

Reverse Transcription PCR (RT-PCR) is applied mainly to examining target genes and detecting fusion transcripts. This technique was done by the production of complementary DNA (cDNA) from the target RNA template using a reverse transcription enzyme. RT-PCR can be combined with other techniques, such as quantitative PCR (qPCR), and this combined technique is referred to as real-time or quantitative reverse transcriptase PCR (RT-qPCR)(11).

Routine PCR amplification of DNA for evaluation of gene fusion is very difficult because much gene fusion happened due to rearrangements of large and different DNA sequences that mainly harbor large intronic (noncoding) DNA regions. The RNA fusion transcript used as a substitute for the DNA rearrangement makes a more reliable PCR amplification procedure and it gives a direct assessment of the amount of fusion transcript present. The fusion of the BCR-ABL1 gene is associated with the formation of the Philadelphia translocation (Ph) and which is the main genetic abnormality in CML and adult acute lymphoblastic leukemia (ALL). Detection of this enables diagnosis and monitoring response to treatment(12).

Multiplex PCR is capable to amplify more than one target sequence in one PCR reaction mixture. This was done by adding multiple specific primer pairs in one reaction mixture. For a good multiplex reaction, the design of specific primer sets is important. It is frequently combined with other PCR techniques such as RT-PCR and qPCR to detect the specific signal associated with each amplified target. After PCR amplification, the DNA containing a mix of amplified sequences can be differentiated by hybridization to fluorescent-labeled probes specific to

different target sequences, separated by size analysis (electrophoresis), or sequenced by next-generation sequencing(13).

Multiple gene shifting is detected at a similar time by using multiplex PCRs. Various mutations of genes that result in neoplasm can be gathered in one multiplex mixture PCR test. Reciprocal translocation of chromosomes 9 and 22 has multiple potential combinations that generate the p190 which is more common in ALL, p210 which is more common in CML, and p230 isoforms. Using multiplex PCR all are detected in one PCR reaction with specific primers to each isoform(13).

Components of Polymerase Chain Reaction (PCR)and Schematic representation of the *ABL* and the *BCR* genes in the t(9;22) are shown in Figures 1 and 2.

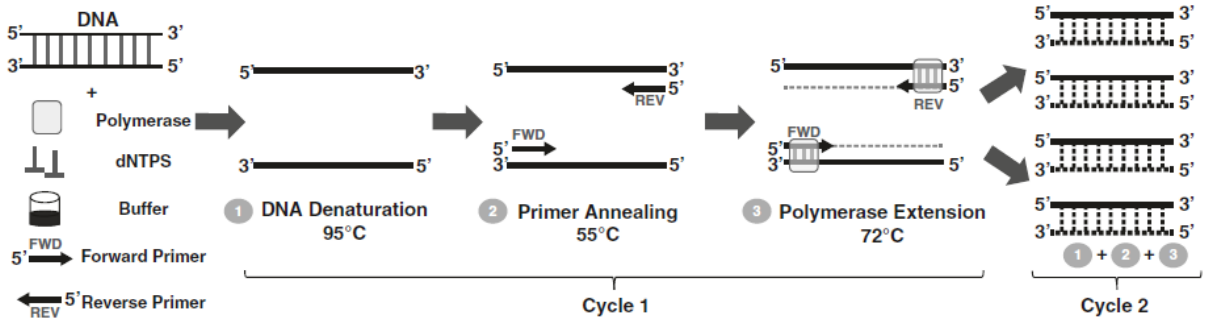


Figure 1. Components of the conventional polymerase chain reaction (PCR) include a DNA template, deoxynucleotide triphosphates(dNTPs)

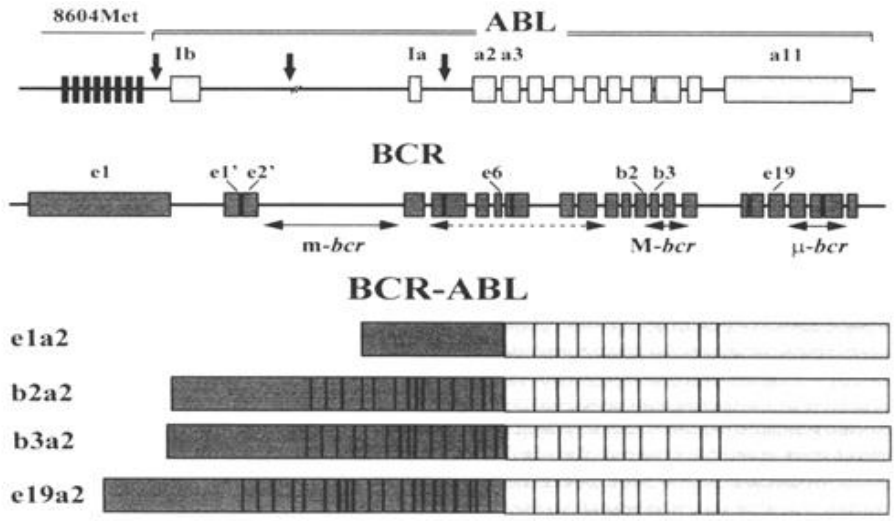


Figure 2. Schematic representation of the *ABL* and the *BCR* genes in the t (9; 22). Exons are represented by boxes and introns by connecting horizontal lines(14).

1.2. Statement of the problem

Translocations of chromosomes were common in patients with hematological malignancies due to genetic instability and can also end up with the inactivation of an oncogene under a new enhancer or promoter or in the generation of a fusion of protein with oncogenic potential. From this chronic myeloid leukemia, one of the hematological malignancies mainly happened in translocation of chromosome 9 with chromosome 22 that results in BCR-ABL1 gene translocation(15).

The BCR-ABL1 fusion protein is a component of an active kinase that can drive uncontrolled proliferation in myeloid precursor cells which is a clinical sign of CML. The structural characteristics of the chimeric BCR-ABL1 protein result in the approval of several tyrosine kinase inhibitors (TKIs) designed to inhibit the intrinsic kinase activity of the ABL1 moiety of the hybrid protein. The capability of these TKI to lower the number of leukemic cells in CML patients needs more sensitive techniques as well as evaluation by molecular monitoring methods to track the dynamics of the disease. Even if there was success behind the targeted therapies in CML, there is a broad challenge of consistently and redundantly managing the level of BCR-ABL1 transcript because of the design and performance characteristics of research-use-only reagents and laboratory-developed tests(2).

The National Institutes of Health Consensus Group recommended the standardization of BCR-ABL1 monitoring using an international scale (IS)(16). According to current developments, a multiplex polymerase chain reaction (PCR) based method of screening helps to detect the presence or absence of unknown gene fusion, which was different from the wild-type polynucleotide sequence of the gene that is isolated from genomic DNA. The multiplex PCR methods of the invention are specifically advantageous in that they permit for identification and characterization of translocations, even against a background containing a vast excess of wild-type molecules. For example, the methods allow for the detection of chromosomal translocations and mapping their breakpoints in samples of genomic DNA containing up to 99 % of wild-type DNA contamination(10).

The result of the international scale (IS) is expressed as a percentage relative to the standardized baseline established in the International Randomized Study of Interferon and STI571 (IRIS) study. Monitoring of *BCR-ABL1* transcripts and its detection limit, particularly after the treatment, needs more standardization which reflects the relatively few *BCR-ABL1* quantification assays available for molecular testing(17). Chronic myeloid leukemia became a

problem for cancer-targeted therapy because of the unprecedented clinical success of the tyrosine kinase inhibitor imatinib. A large number of CML patients who are in the chronic phase and are treated with imatinib achieve durable remissions and their survival is not quite different from that of the general population. However, a fraction of patients were intolerant or resistant to imatinib(18). Around half of all individuals with CML develop disease relapse shortly after discontinuation of TKI treatment(19). This shows that a substantial number of quiescent CML cells remain at the time of treatment cessation, frequently giving rise to relapse.

In addition to the well-established methods and certified commercial techniques available for high sensitivity monitoring of *BCR-ABL1* transcripts,(20) application of DNA-based monitoring may be a useful adjuvant tool to aid treatment decisions(21). In Ethiopia, there was a challenge of a limited facility for doing the quantitative PCR. There was no study conducted for evaluations of multiplex PCR as potential prognostic tools for CML patients as compared to other methods used for screening CML due to resource limitations. In this study, we ratified how multiplex PCR can be utilized as potential prognostic tools for CML patients in a resource-limited setting at TASH and show how they respond to treatment provided for them in terms of molecular response related to their base pair.

1.3. Significance of the study

The identification of BCR-ABL fusion genes was critical for precise diagnosis, risk stratification, and therapy scheme selection and considering drug discontinuation for patients relax for the prolonged molecular response. The other important significance of this study is it helps to determine whether the patients who were in follow-up clinics especially those who were taking treatment for three and six months or more have good molecular responses for treatments they took. Hematological response, then Cytogenic response, and then MMR, is expected to achieve within a specific treatment milestone period. So, the advantage of qualitative multiplex-PCR is to know the type of transcript variation in CML patients and as prognostic tools for patients taking the treatment to know their molecular response for the treatment they are taking. The findings will also set a critical milestone for treatment response unless it needs to enforce the physician to change the medication regime and to identify if there were other related factors are there or not.

Even if there is real-time quantification PCR to quantify the BCR-ABL1 translocation, it was costly, and patients could not afford well to have this test all the time during their visit to the hematology clinic at TASH. Plus there were limited resources to have the kit used for the real-time quantification PCR. Thus, the presence of the reverse transcriptase multiplex PCR as a potential prognostic tool for CML patients which detect up to 0.31 IS% was very important to reduce challenges faced by patients to have this test. The physicians had not known very well the treatment outcome of their patients because they used hematology response for treatments because of resource limitation for having quantitative PCR to be done abroad. In our country, this study benefits patients and clinicians to monitor the clinical outcome of their patients for treatment provided to them especially for those taking treatments for more than one year, and also helps them to know which oncogene was transcribed for causing their CML.

2. Literature review

2.1 Pathology of chronic myeloid leukemia.

The BCR-ABL gene is a fusion oncoprotein that happened due to the Philadelphia (Ph) chromosome which results from a reciprocal translocation involving the long arms of chromosomes 9 and 22, t(9;22) (q34;q11) in hematopoietic stem cells(HSC). The BCR-ABL gene fusion on the Ph chromosome happens to head to tail with the 5' end of BCR coupled to the 3' end of ABL(22). There is no perfect reason behind translocations of these genes; however, most suggested it probably occurred because of continuous chromosomal break and repair during mitosis which is enhanced by ending proximity chromosomes 9and 22in the interphase nucleus(23). This translocation is the most common type of cytogenetic abnormalities in hematology disorders(24) which is found in 95% of chronic myeloid leukemia patients.

At the stage of the molecular level, t (9; 22) is characterized by the fusion of the proto-oncogene ABL (9q34.1) and the breakpoint cluster region (BCR) at band q11.2 on chromosome 22. This gives rise to a chimeric BCR/ABL gene, which is translated into a fusion protein with a high tyrosine kinase activity(25). The main importance of the tyrosine kinase enzyme is to catalyze the transfer of a phosphate group from adenosine triphosphate (ATP) to target proteins that play a crucial role in cell signal cascade that regulate cell proliferation, differentiation, anti-apoptotic signal transduction, and programmed cell death(26).

Increased TK activity of p210 bcr-abl came up with phosphorylation of several cellular substrates and in autophosphorylation of p210 bcr-abl that induces the engagement and binding of numerous adaptor particles and proteins. Uncontrolled production of TK results in the hindrance of basic cellular processes, like control of cell proliferation, differentiation, adhesion, and apoptosis(27). This results in leukemic progenitors' growth-stimulating factors, such as IL-3 and granulocytes colony-stimulating (GCS) factor (28).

The breakpoint for BCR is located to a 5.8-kb (kilobase) area between exon e12-e16 known as the major breakpoint cluster region (M-bcr) for Ph-positive CML and one-third of B – cell acute leukemia (B-ALL). Alternative interlacing results in the fusion transcripts either b2a2 or b3a2 joint that give 210 kDa proteins (p210 bcr-abl1)(29). Not often in 2/3rd of the patients with Ph^+ B-ALL and in some CML, the BCR breakpoint localized to 54.4-kb area between exon e2' and exon e2 called minor breakpoint cluster region or m-bcr, that give an e1a2 transcript

which translated into P190 BCR-ABL. Whereas in the case of ABL the breaking point takes place downstream exon Ia, up the stream of exon Ib, or between exon Ia and Ib(25)another breakpoint cluster region that generates 230-kDa fusion protein (p230 BCR-ABL), is mainly found in chronic neutrophilic leukemia (30).

2.2. Diagnosis of chronic myeloid leukemia.

Around forty percent of chronic myeloid leukemia patients are diagnosed unfortunately without having awareness about their disease during a routine blood test. The sign and symptoms of the patients are splenomegaly, abdominal discomfort, night sweats, weight loss, and weakness(31).

The chronic phase of CMLis mostly differentiated by a higher white blood cell count consisting primarily of differentiated myeloid cells (metamyelocytes, myelocytes, bands, and neutrophils), basophilia, and in certain patients increased platelets and splenomegaly. The diagnosis is done by the analysis of blood either by qualitative or quantitative reverse-transcription polymerase chain reaction (QPCR) or interphase fluorescence *in situ* hybridization (FISH) to identify the BCR-ABL translocation. Three bcr-abl protein reports have been discovered; these are, p210, p190, and p 230 which are identified based on their molecular weight(32). P 210 KDa is the dominant protein in CML patients(33).

The diagnosis of CML which is sometimes done from peripheral blood and bone marrow examinations is an important phase for CML which will be distinguished in chronic phase (CP), accelerated phase (AP), and blast crisis (BC) phase CML. Knowing the characteristics consistent with AP or BC disease at diagnosis is important for prognosis because these patients have inferior therapeutic responses(34).

2.3. Treatment response for tyrosine kinase inhibitors (TKI)

It is very crucial to understand how the response to therapy is measured in CML patients. Because, the sensitivity of each test, hematologic response precedes cytogenetic response, and cytogenetic response, in turn, precedes major molecular response. Complete hematologic response (CHR) in the peripheral blood occurs when WBC <10 X10^9/L with immature granulocytes, < 5% basophils, platelet count< 450 X10^9/L and spleen size not palpable(35).The Cytogenetic responses are measured by the percentages of cells by G-banding of metaphase preparations that remain Philadelphia chromosome-positive and are classified as complete (0%), partial (1–35%), minor (36– 65%), minimal (66–94%), or lacking (> 95%) (36).

A major cytogenetic response (MCyR) encompasses both CCyR and partial cytogenetic response. The interphase FISH using probes directed against BCR-ABL breakpoints is a more sensitive tool for monitoring disease than metaphase cytogenetic preparations(37, 38).

Tyrosine kinase inhibitors play the main role in the clinical management of patients with chronic myeloid leukemia (39). To evaluate response to TKI therapy quantitative and qualitative monitoring of the fusion transcript BCR-ABL1 (breakpoint cluster region–c-abl oncogene non-receptor tyrosine kinase (BCR-ABL1) transcripts in peripheral blood by reverse transcription-quantitative PCR (RT-qPCR) is the gold standard. The log reduction of transcripts amount at the specific time is prognostic and predicts patient treatment failure. This monitoring method has been adopted into the ELN (EuropeanLeukemia Network) guidelines. Patients are classified as good responders when the percentage of BCR-ABL1IS(%BCRABL1IS) is10% at 3 months after treatment initiation followed by 1% at 6 months and 0.1% at12 months (major molecular response (MMR). The performance of this BCR-ABL1 monitoring test meets all of the clinical guideline recommendations for sensitivity and IS reporting for the management of chronic myeloid leukemia patients(40).

Multiplex reverse transcription-quantitative real-time PCR (Multiplex RT-qPCR) is developed for the detection of at least 14subtypes of BCR-ABL fusion genes. The modern multiplex RT-$_q$ PCR is capable of detecting BCR-ABL fusion genes with sensitivity up to 10-10^6 copies (7). Treating chronic myeloid leukemia needs quantitative polymerase reaction for monitoring BCR-ABL1 on an international scale (IS)(41).

In Ethiopia, data on the national prevalence and incidence of CML patients are lacking despite the increasing rates of patient attendance and medical admission. Through the support from Gleevec International patients Assistance Program (GIPAP), CML patients are receiving treatment and are documented at TikurAnbessa Specialized Hospital (TASH); the only center in the country treating CML. According to this document, more than 1400 CML patients had been treated by Imatinibfrom January 2004 to December 2017. The drug is available free of charge, regardless of financial status, for the treatment of all patients with CML. There were 920 actively treated CML patients in the unit since January 2018 (Gleevec International Patient Assistance Programme, 2018). Also, though there was no formal survival study done on Ethiopian CML patients; based on physicians' reports and witnesses, there are patients who survived ≥14 years through Imatinib treatment since confirmed diagnosis as compared with a maximum of 2-years

survival by conventional drugs either by Busulphan or hydroxyurea (HU) as per physicians report.

A prospective cohort study was conducted at TikurAnbessa Specialized Hospital from October 1, 2016, to November 30, 2017, that aimed at assessing treatment outcome and adherence to Imatinib in patients with CML treated at the outpatient hematology unit. One hundred forty-seven patients were newly screened for eligibility to engage in the study. Participants' median age at the time of diagnosis was 36 years (Ranged: 14-74); with 95(64.6%) of them in the age group of ≤40 years. Male comprised 59.2%. Apart from the lost-to-follow-up (n=3), 132(91.7%) of the patients achieved complete hematologic remission with a median treatment response period of 6-weeks. Peripheral blast count ≥5 %(AOR=0.33, 95%CI: 0.16, 0.79) was found to be predictors for CHR failure, whereas adherence (AOR=8.60, 95%CI: 4.32, 11.10) was positively associated with CHR. Low platelet count at Imatinib initiation (AOR=5.3, 95%CI: 2.35, 8.7) and being female (AOR=2.82, 95%CI: 1.32, 4.94) were significantly associated with treatment discontinuation and dose decrement due to adverse drug events. The adherence rate to Imatinib was found to be 55.6%(42).

A retrospective descriptive study aimed to determine the distribution and spectrum of various hematological malignancies encountered in Gondar University Hospital, North West Ethiopia from January 2008 to December 2011. In those admitted with the diagnosis of hematological malignancies, 67 patients were admitted and the mean and median age of patients was 42and 45 years, respectively. The result shows Non-Hodgkin's lymphoma (NHL) comprised 22/67 (32.8%) of all hematological malignancies, followed by 17/67 (25.4%) chronic myeloid leukemia (CML) and 13/67 (19.4%) chronic lymphocytic leukemia (CLL). Among patients with CML, 12/17 (71%) were in the chronic phase, 4/17 (23%) in the accelerated phase, and 1/17 (6%) in the blast phase on admission. The majority of CLL patients had advanced disease on admission with 9/13 (69%) Binet C and 10/13 (76%) (Rai stage III and IV)(43).

A retrospective descriptive study was carried out on the distribution of various hematologic malignancies among patients who have received bone marrow examination, in the Eritrean National Health Laboratory from October 2015 to July 2017. The result indicates that from 207 patients for whom marrow aspiration is done, 52 patients were hematologic malignancy cases. The male-to-female ratio was 1:1. The age of the patients ranged from 2 to 80 years. Of the 52 patients, 19 were less than 20 years of age and the remaining 33 were 20 years and above. Acute leukemia was the most common hematologic malignancy in the study area. It

affected 18 of the cases followed by chronic myelogenous leukemia, chronic lymphocytic leukemia, myelodysplastic syndromes, multiple myeloma, and myeloproliferative neoplasms. More than two-thirds of the patients had a total leukocyte count greater than 10,000/μl(44).

Another descriptive cross-sectional study was carried out in 112 CML patients who were attended different clinics in Khartoum state, Sudan from February 2007 to December 2010. Transcript analysis of 109 samples showed that 32.1% (35/109) expressed one or both of the P210 BCR/ABL rearrangements (b2a2 and b3a2). Of those 35 (32.1%), 21 patients expressed b2a2, 6 expressed b3a2, and 8 expressed both transcripts b2a2/b3a2. The remaining 74 patients revealed transcripts combination of P190 BCR/ABL and P210 BCR/ABL (e1a2/b2a2/b3a2), of which 19 patients had all the transcripts (e1a2/b2a2/b3a2), 33 revealed 2 transcripts (e1a2/b2a2), and the remaining 22 patients had (e1a2/b3a2)(45).

Retrospective analysis of clinical specimens in line with the policies of Stem cell and Cancer IRB and following the Declaration of Helsinki was performed in Indonesia. Separate biotinylated RT-PCR primers were designed to amplify specific BCR-ABL transcripts and JAK2 V617F mutant alleles. Specific hybridization of RT-PCR products with arrays of membrane-bound probes followed by colorimetric development would allow simultaneous visualization of BCR-ABL and/or JAK2 mutant transcripts in a given specimen. Their result indicated that the limit of detection (LOD) or analytical sensitivity of the RDB method using cDNA specimens was 0.5% and 6.25% in detecting BCR-ABL and JAK2 mutant transcripts, respectively. Diagnostic specificity and sensitivity to detect BCR-ABL and JAK2 were 100% and 92.3% (N= 38); 100% and 100% (N= 27), respectively. RDB also detected BCR-ABL transcripts in 22% of JAK2 V617F mutation-positive samples (N=14)(46).

Another study with chronic phase CML patients enrolled in consecutive or parallel clinical trials was conducted at the MD Anderson Cancer Center, USA, using TKI as front-line therapy from July 31, 2000, to September 10, 2013. The study involved 481 patients with chronic phase CML expressing different types of BCR-ABL transcripts. Off those, two hundred patients expressed e13a2 (42%), 196 (41%) expressed e14a2, and 85 (18%) expressed both transcripts. The proportion of patients with e13a2, e14a2, and both achieving complete cytogenetic response at 3 and 6 months was 59%, 67%, and 63% and 73%, 81%, and 82%, respectively, whereas major molecular response rates were 27%, 49%, and 50% at 3 months, 42%, 67%, and 70% at 6 months, and 55%, 83%, and 76% at 12 months, respectively. The median (international scale) levels of transcripts e13a2, e14a2, and both at 3 months were

0.2004, 0.056, and 0.0612 and at 6 months were 0.091, 0.0109, and 0.0130, respectively. In multivariate analysis, e14a2 and both predicted optimal responses at 3, 6, and 12 months. The transcript type also predicted for improved probability of event-free (P = .043; e14a2) and transformation-free survival (P = .04 for both). Relative to e13a2 transcripts, patients with e14a2 (alone or with co-expressed e13a2) achieved earlier and deeper responses predicted for optimal European Leukemia Net (ELN) responses (at 3, 6, and 12 months) and predicted for longer event-free and transformation-free survival(47).

A cohort study conducted at Departments of Medical Oncology, Sher-i-Kashmir Institute of Medical Sciences (SKIMS) and Clinical Hematology, SKIMS in India recruited 42 CML patients including 18 males (42.85%) and 24 females (57.14%). Patients aged from 7 to 75 years and were followed up for the response to imatinib treatment. Patients were subjected to Multiplex RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) and were all found to harbor either e13a2 or e14a2, which could be analyzed by a single Taqman probe-based quantification kit (Geno-Sen's) to quantify the BCR-ABL transcript load. The Multiplex RT-PCR and Peripheral blood cytogenetics providing specific and sensitive detection of BCR-ABL fusion transcripts and metaphase signal load, respectively, were used as parallel reference tools to authenticate the q-PCR findings. There was 100% concordance between the multiplex RT-PCR and the q-PCR as every transcript load above 0% reflected as positive (+ve) RT-PCR assay for that transcript(4).

A cross-sectional study was performed on 85 cases (PB & BM) of CML patients at the molecular pathology laboratory of Ghaem Hospital, Mashhad, Iran in 2012-2013. Fusion of b3a2 was detected in 53 (62.35%) patients, b2a2 in 25 (29.41), e1a2 in 1 (1.17%) and co-expression of b3a2 and e1a2 in 6 (7.05%) patients. There were significant differences between the mean age in patients who were b3a2 positive (44.07 years) and b3a2 negative group (50.35 years). However, no significant differences were seen between sex and b2a2 (P=0.61), b3a2 (P=0.79) and e1a2 (P=0.20).This study showed higher frequency b3a2 than b2a2 and e1a2 transcripts in CML patients in Northeast Iran and there was no association between e1a2 transcripts frequencies and monocytosis in peripheral blood(48).

A study designed to compare the feasibility of the one-step multiplex RT-PCR with the conventional multistep RT-PCR technique from, BM or PB samples for diagnosis from 548 patients at the Catholic University of Korea between November 1999 and May 2005. Patients were referred with a diagnosis of Ph+ CML and their median age was40 years (range 16–76

years). Of the patients, 234 (42.70%) were women and 314 (57.30%) were men. The sensitivity of multiplex RT-PCR and nested RT-PCR was measured for each transcript type, and the results are showing, the sensitivity of multiplex RT-PCR was similar for all transcripts. Most patients (538/548, 98.18%) expressed one p210 BCR-ABL rearrangement, and of the cases positive for p210, 364 patients (67.66%) corresponded to b3a2, and 174 (32.34%) corresponded to b2a2. The b3a2 was expressed in 64.82% of female patients and 53.75% of male patients, and b2a2 was expressed in 33.22% of female patients and 29.88% of male patients. No differences were observed between women and men. In the multiplex RT-PCR, the product of unexpected size not detectable in conventional RT-PCR was detected in 1 patient, and e1a3 was confirmed by the direct sequencing of this product(49).

A retrospective study was carried out at the Department of Haematology, Christian Medical College, Vellore, India, between January 2005 and June 2016. The study was designed to determine the type and frequency of BCR-ABL1 fusion transcripts. Of the 1260 CML patients screened, 1188 (94.2%) expressed one of the two major BCR-ABL1 transcripts. There were 756 patients with e14a2 (60%) and 432 patients with e13a2 (34.3%) transcripts, respectively. Although the number of male patients was almost double the number of female patients (males N = 823, females N = 437), there was no difference in the frequency of the BCR-ABL1 fusion transcripts between male and female patients. Fifteen (1.1%) patients had an isolated e1a2 transcript, while 23 (1.8%) and 26 (2.0%) patients had e1a2 in combination with e14a2 or e13a2, respectively. Other rare transcripts identified includes e19a2 (4 cases) and e14a3 (4 cases). Eight of the 12 (67%) patients with e1a2 transcript had disease progression (three developed myeloid blast crisis, three had lymphoid blast crisis, and two patients progressed from CML-CP to CML-AP)(50).

In general, different kinds of literature recommend molecular analysis of BCR-ABL fusion by polymerase chain reaction (PCR) can confirm the presence of the Philadelphia chromosome because PCR performs at higher sensitivity and specificity. Furthermore, quantification of ABL fusion genes by PCR serves as a baseline measurement for continuous monitoring of the disease.

3. Objectives

3.1. General Objective

- To evaluate the multiplex PCR as a potential prognostic tool for chronic myeloid leukemia (CML) patients in a resource-limited setting at TASH, Addis Ababa, Ethiopia from January 2020 to May 2021.

3.2. Specific Objectives

- To evaluate multiplex RT- PCR for detection of BCR-ABL translocation in comparison with the standard prognostic tool, quantitative multiplex RT-PCR.
- To assess the association of specific transcripts with the demographic and clinical status of the patients

4. Materials and Methods

4.1. Study Area

The study was conducted at TASH, which was established in 1972. Tikur Anbessa Specialized Hospital, located in the nation's capital Addis Ababa, is Ethiopia's largest referral hospital in the country. The hospital was given to Addis Ababa University by the Ministry of Health as the main teaching hospital in the school of medicine. TASH delivers diagnosis services and treatment for approximately 500,000 patients per year in its 20 outpatient specialty clinics, inpatient, and emergency unit. From those, the hematology clinic is one of the largest units which deliver comprehensive specialty service for leukemia patients mostly CML patients. Three to five newly diagnosed patients per week and a total of more than 60 CML patients visit the clinic. Outpatient services to CML patients were given in the clinic four times per week. All CML patients took Imatinib free of charge from the oncology pharmacy.

4.2. Study Design and Period

A hospital-based cross-sectional study design was conducted from January 2020 to May 2021at TASH.

4.3. Population

4.3.1. Source of population

All CML patients attending Tikur Anbessa specialized Hospital in the hematology unit during the study period were included.

4.3.2. Study population

All eligible volunteering patients aged 18 years and above who were newly diagnosed with chronic myeloid leukemia and patients who were in the follow-up clinic of hematology-oncology for treatment of CML at Tikur Anbessa Specialized Hospital during the study period.

4.4. Inclusion and Exclusion criteria

4.4.1 Inclusion criteria

- All patients were newly diagnosed with chronic myeloid leukemia or were in the follow-up clinic of hematology-oncology.

- Willing to participate
- Patients who were positive for the Philadelphia chromosome and their age was equal to 18 or above at Tikur Anbessa Specialized Hospital during the study period.

4.4.2. Exclusion criteria

- Patients who had severe anemia
- Patients who had recently been drawn blood (to avoid repeated/redundant vein puncture)

4.5. Study Variables

4.5.1. Dependent variables

- Multiplex RT-PCR result (either major/minor Transcripts or bcr alone
- BCR-ABL transcript (Qrt-PCR) in % International scale(IS)

4.5.2. Independent variable

Socio-demographic variables

- Age
- Sex
- Residence
- Types of treatment and its dosage
- Follow up duration
- Sokal score at baseline.

Clinical data at baseline and follow-up, such as

- Spleen size (cm)
- Splenomegaly present or absent
- Hepatomegaly
- Hemoglobin, g/dl
- Total leukocyte count (TLC) $x10^3/mm^3$
- Platelet count $x10^3/mm^3$
- Peripheral blood blast count in % at baseline
- clinical stage of CML (chronic, accelerated, and blast phase)

4.6. Sample Size Determination

A convenient sample technique was used for this study. Accordingly, 114 study participants who fulfill the eligibility criteria during the study period were included.

4.7. Data quality assurance

Pre-analytical phase

Aseptic technique was followed at all times and each sample was treated as it was infectious. The sample was collected from TASH in a 5 ml EDTA tube and transported to AHRI in the icebox. All the preanalytic phase precautions were made to ensure the quality of the reagents used in the analysis. To ensure the quality of Red blood cells lysis, a one-times NH4CL concentration was prepared by weighing 33.2g NH4CL, 0.1488g EDTA, and 4g $KHCO_3$ and put all the powders into 500 ml Erlenmeyer flask, and water is added up to 500 marks little by little until completely dissolved. This means 8x concentrations because under normal conditions we diluted it up to 4000ml (4000/500=8). Eight times concentration was to prevent it from contamination. To prepare a working solution, 100ml of the concentrate (8 xs) was taken and 700ml water was added. I.e. total of 800ml with (1x) used to lyse RBC. The quality of the RNA sample greatly affects the results of this test. To minimize the risk of degradation of RNA by ribonucleases, the cells were lysed in a denaturing solution containing guanidinium isothiocyanate (GTC)] that was prepared from GTC-Buffer (10ml 0.5M Sodium Citrate, pH7 + 1g SARCOSYL + 94.52g GTC fill up to 200ml with DEPC-H_2O); 4M GTC,25mM Sodium citrate (pH 7),0.5% Sarkosyl), for using the solution 8microliter/ml β-Mercapto-ethanol was added. The solution was stable for 4 weeks and Kept in ice. More detail of the protocol is written in annex I.

Analytical phase

All procedures in this phase were performed strictly following protocols. Eye protection and disposable gloves were worn during all steps of the assay for doing multiplex PCR.RNA was extracted by Qiagen RNA extraction kit and the quality of the RNA was read using Nanodrop. cDNA synthesized in volume 40µlusing reverse transcriptase enzyme 8µl with 32µl RNA sample. Multiplex PCR was run with master mix and synthesized cDNA for amplification of DNA from the sample with control materials. That is used for electrophoresis to see the fragments in their respective base pair. The fragmented base pair was seen against the control material K562 for b3a2, BV-172 for b2a2, and SD-1 for e1a2 transcripts that had been seen in the gel-electrophoresis image. A more detailed protocol was written in annex I. A Set of micropipettes, aerosol barrier pipette tips, disposable gloves, and clean lab coats has been available in each of the four rooms. The work was organized so that mixes and reaction products

only move in the direction from Master Mix room to cDNA room to PCR room to Gel electrophoresis room. Never move mixes or reaction products in the opposite direction.

Post-analytical phase

The remaining specimen after completion of the procedure was stored appropriately to be used for different test assays and the data collection sheet was assessed for their completeness. Generally, the procedure was done according to a well-reviewed standard operational procedure (SOP) addressing the pre-analytical, analytical, and post-analytical phases of each assay.

4.8. Measurement and Data collection

4.8.1. Data collection procedure

All patients fulfilling the eligibility criteria were recruited consecutively at the hematology clinic. A pre-tested, semi-structured questionnaire and data abstraction format (Annex II) were used to extract information from the patients and medical records, respectively. After obtaining informed consent to participate in the study, information on socio-demographic characteristics was gathered using a questionnaire. Data on socio-demographic variables and other necessary information including laboratory investigations were collected from patient's medical records (charts) using structured formats. All the data collection formats were filled in the Hematology-Oncology center of TASH.

4.8.2. Sample collection and processing

The peripheral blood sample was collected in a 5ml EDTA tube from new and follow-up leukemia patients at TASH and processed on the same date at AHRI. Whole blood was mixed with 40 ml of 1 times NH4CL solution and incubated for 25 minutes on ice, and the WBC pellet was washed with ice-cold PBS twice. After counting the WBC, ~10 million cells using the WBC counting chamber under microscopy cells were lysed with GTC+β-Mercaptoethanol (reagent preparation is annexed) and stored at -80oC until RNA extraction will be done.

4.8.3. RNA extraction and cDNA synthesis

RNA was extracted from peripheral blood specimens using the standard method as in the manufacturer’s protocol of RNeasy Mini Kit (QIAGEN 74106). After the RNA was extracted, quantity and quality were assessed using a Nanodrop OD of 1.0 at 260 nm which was equivalent

to approximately 40 µg/ml single-stranded RNA. An OD260/OD280 ratio between 1.8 and 2.1 is indicative of highly purified (51).

cDNA synthesized using Superscript. First, the measurement of RNA was done in RNA-free water buffer then, transcribed into cDNA in a reaction mixture comprising random hexamers, reverse transcriptase, RNase inhibitor, and dNTPs by using a thermocycler. The complementary DNA was a template for two multiplex Master PCR amplification using a hot start Taq DNA Polymerase and specific PCR primers. The fusion gene had many breaking points. Primers of the PCR were designed to bind at a position enabling them to screen for all these breaking points. In case if a Master PCR reaction is positive for a translocation-specific amplicon the cDNA is also used as a template for Split-out PCR reactions. PCR products were visualized by agarose gel electrophoresis.

4.8.4. Multiplex RT-PCR

Multiplex RT-PCR were performed by using, 2µL of cDNA synthesized from RNA and control cDNA prepared from cell lines (K562, b3a2; BV173, b2a2; SD1, e1a2) were mixed with 21µL of PCR-Mix containing 2.3µl 10x Buffer, 2.3µl MgCl2 [25mM], 0.184µl dNTPs [25mM], 0.46µl primer mix (BCR R1, BCR F3, BCR F4, ABL R2 [12.5µM each]), 15.656µl of water, and 0.1µl Taq-polymerase 5units/µl. For each primers sequence used is

PCR BCRF3: ACCGCATGTTCCGGGACAAAAG (BCRExon1),

BCRF4: ACAGaATTCCGCTGACCATCAATAAG (BCR Exon 13)

BCR R1: ataggaTCCTTTGCAACCGGGTCTGAA (BCR Exon 21), and

ABL R2: TGTTGACTGGCGTGATGTAGTTGCTTGG (ABL Exon 3).

Then all this was submitted to 35 cycles in a thermo cycler. Cycle times and temperatures for denaturation, annealing, and synthesis was 30 s at 96 °C, 50 s at 60 °C, and 1 min at 72 °C, respectively, followed by a 10 min extension at 72 °C.

To possibly amplify the entire most possible Bcr-Abl1 fusing gene, four types of primers (two forward and two reverse) were utilized using standard multiplex PCR protocol. The amplified product was run using 2% agarose gel electrophoresis in TEA buffer at 80V for 60 minutes, parallel with 100bp ladder, and control cDNA prepared from cell lines (K562, b3a2; BV173, b2a2; SD1, e1a2). The two molecular variants yielded products of 310 bp (b2a2) and 385 bp (b3a2) respectively. The e1a2 product is 481 bp while the *BCR* gene808was used as internal control with a PCR product of 808 bp (52).

4.8.5. Real-Time Quantitative Polymerase Chain Reaction (qPCR)

TaqMan™ reporter assays that avoided the problem of non-specific fluorescence by using a fluorescently labeled probe that was hybridized to a region within the target amplicon sequence was used (53). The probe had a 50 fluorescent label and a 30 quencher molecule close, preventing fluorescence under fluorescent resonance energy transfer. The primers and the probe bound to cDNA during the PCR annealing stage. At any time next stage extension, Taq polymerase cleaved the 50 fluorophores from the probe, which enabled detached from the quencher and allowed it to fluoresce. Amplification was thereby measured by the accumulation of the fluorophore during each extension stage, with the intensity of the fluorescence proportional to the number of amplicons, and the accumulation was related to the quantity of the target, that was, BCR-ABL1, in the starting sample.

The number of transcripts was calculated by comparison with standard curves generated using serial dilutions of BCR-ABL1 cDNA at known concentrations, which were included in each run dual times. Certified reference material was available for the BCR-ABL1 test which consists of plasmids at known concentrations that contain both the BCR-ABL1 and ABL1 genes. The standards cover a copy number range from 10^1 to 10^6 copies per µl(54). Dual q-PCR data were also normalized against a reference gene (usually ABL1, BCR, or GUS) to take into account the different concentrations of RNA in the initial clinical sample, and multiplied by conversion factor settled for institutions 0.542. The cycle threshold q PCR was settled as 50 ^{o}c for 2 minutes, 95 ^{o}c for 10 minutes, and 95 ^{o}c for 15 seconds times 50x, and 60^{o}c for 1 minute. Multiplex-qPCR reactions were performed simultaneously in the same well when the probes have different color fluorescent labels. Reagent, primer, and probe used dual q-PCR are written in Table 1. As an internal quality control, 5% of the samples were repeated to check the reproducibilities of the test result.

Table 1: MasterMix: Taqman Universal PCR Mastermix No Amperase UNG-4324081, Applied Biosystem

Primer and Probe			
	Primer		Probe
BCR-ABL:	Forward	Reverse	
e13a2/e14a2	ENF-501	ENR-561	ENP_541 FAM/TAMRA
ABL:			

	T494 ENF_1003	T495 ENR_1063	ENPR 1043 FAM/TAMRA	

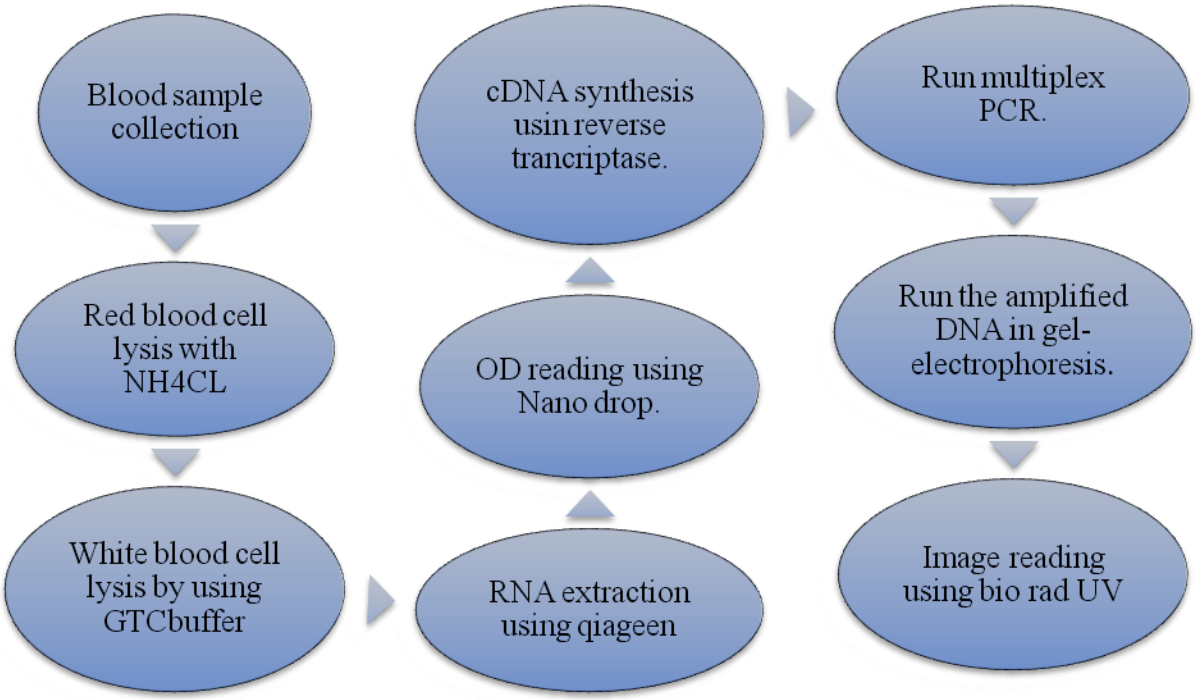
Table 2: Reagents used for quantitative PCR

Reagent	Concentration	Volume1x	Final concentrations	Volume 10x
H2O, PCR grade		5.50 µl		55.0 µl
Probe	10µM	0.50 µl	2 µM	5.0 µl
Primer forward	10 µM	0.75 µl	3 µM	7.5 µl
Primer reverse	10 µM	0.75 µl	3 µM	7.5 µl
PCR Master Mix Universal	-	12.5µl	-	125.0µl
Volume pre tube		20.0 µl	-	200.0 µl
cDNA		5.0 µl		
Total volume		25 µl		

4.9. Data Analysis and Interpretation

Data were entered and analyzed by using SPSS version-27. Descriptive statistics including frequency, median, range, andx^2were used to summarize socio-demographic data, clinical and treatment-related characteristics, and evaluate the distribution of responses. The ROC curve analysis was performed by excel 2007 Microsoft ware to see the agreement between multiplex RT-PCR and quantitative RT-PCR in terms of sensitivity and specificity to determine the cut-off value for knowing the limit of detection for multiplex RT-PCR. And, Sokal score was calculated by using the formula exp(0.0116 ×(age[years]-43.4)) + (0.0345 ×(spleen size[cm]-7.51) + (0.188 × ((platelets [10^9/L]/700)^2 -0.563)) + (0.0887 × (blasts[%] -2.10)) using excel 2007 Microsoft to predict prognosis at the time of CML diagnosis before starting treatment. A P-value less than 0.05 was considered statistically significant.

4.10. Work flow chart.



4.11. Ethical Considerations

Before conducting the study, the protocol was reviewed and ethical approval was obtained from the Department of Medical Laboratory Science, College of Health Sciences, Addis Ababa University, and the 'Institute Ethics Committee' (IEC). And also, an Official letter was written asking permission for the Department of Internal Medicine in TASH for doing the research there and for AHRI for performing laboratory activities there. Each patient was informed about the objective of the study, procedures of selection, and assurance of confidentiality was maintained. Individuals were informed that they would not receive any monetary incentive for participating in the study. The collected data were secured in a lockable cabinet; no identifiers were used and data was analyzed in aggregate to maintain confidentiality and anonymity of information, and finally, the study outcome was delivered to the Hospital.

4.12. Operational Definition

Chronic phase: four to five years manifestation by myeloid hyperplasia with the domination of mature granulocyte.

Accelerated phase: with a short time of duration at which myeloid element loses their capability to differentiate. Blasts in blood or marrow 10-19%, and Basophils in blood greater or equal to 20% WHO criteria(55).

Blast phase: inevitably acute leukemia of myeloid (70%) or lymphoid (30%) phenotype. Blasts in blood or marrow greater or equal to 20%, and extramedullary blast proliferation, apart from spleen large foci or clusters of blasts in the bone marrow biopsy are seen(55).

Complete Hematologic Response (CHR): White blood cell count <10,000/μL with no immature granulocytes and <5% basophils on the differential, platelet count <450,000/μL, and spleen not palpable.

Molecular Response: Assessed according to the International Scale (IS) as the ratio of BCR-ABL1 transcripts to ABL1 transcripts or other internationally recognized control transcripts.

Sokal Score*:* Developed to aid in prognosis and predict response to treatment with conventional chemotherapy (e.g., busulfan)

IS %: International scale in percent for reporting for the management of chronic myeloid leukemia patients

DM^{4}, $DM^{4.5}$: Deep molecular response for BCR-ABL less than 0.01% IS

4.13. Dissemination of Result

The result of the study will be submitted to the Department of Medical Laboratory Sciences, College of Health Sciences, and Hematology-Oncology Clinic, School of Medicine, Addis Ababa University, and AHRI. The final result will be presented at conferences and published in peer-reviewed journals.

5. Result

5.1. Socio-demographic characteristics of study participants

A total of 114 study participants were enrolled in this study. From these 31 patients were confirmed for Philadelphia chromosome-positivity, but treatment naïve, and 83 were on treatment (follow-up). The socio-demographic characteristic is shown in Table 3. For all patients, their diagnosis was confirmed by clinical data and cytogenetic analysis when they were positive for Philadelphia chromosomes at baseline.

Table 3: -Socio-demographic characteristics of new and follow-up CML patients at Tikur Anbessa Specialized Hospital, Addis Ababa (n=114).

Variable	New case	Follow up
Age		
Minimum	20	19
Maximum	65	83
Median	35	35
Sex		
Male	16(51.6)	56(67.5)
Female	15(48.4)	27(32.5)
Residence		
Addis Ababa	5(16.1)	21(25.3)
Benishangul	2(6.5)	2(2.4)
Oromia region	11(35.5)	28(33.7)
Tigray	2(6.5)	2(2.4)
South nation nationalities	5(16.1)	15(18.1)
Amhara	5(16.1)	14(16.9)
Gambella	1(3.2)	-
Somali	-	1(1.2)

5.2. Baseline clinical and hematological characteristics of study participants

At baseline, the median and range of blast count were 4.0(0-45) and spleen size 14.75 cm (4-30). As shown in Table 4 below, 18.5% had comorbidity, 98.2% had splenomegaly, 49.1% had hepatomegaly, 47.4% had spleen size>15 cm and Sokal score of >1.2 (high risk) was seen in 64.6% patients. Sokal score was low risk in 9(7.8) and intermediate-risk 32(27.6). The median

and range of WBC was 229.93(26-642 x 10^9/L),PLT 344.50(35-1132x 10^9/L), HGB 10.30(5.7-15.4gm/dL) during their first visit to TASH.As shown in Figure 3, 106/114 (93.0%) patients were in the chronic phase, while 4 were in the accelerated phase and 4 were in the blast crisis phase.

Table **4** shows baseline clinical data for the study participants.

Table 4: Baseline clinical and hematological characteristic of study participants

Characteristics	Frequency	Percentage
Any co morbidity		
Yes	20	17.4
No	94	81.7
Splenomegaly		
Yes	112	98.2
No	2	1.8
Hepatomegaly		
Yes	56	49.1
No	58	50.9
Sokal score		
<=0.8 low risk	9	7.9
0.8-1.2 intermediate risk	32	28.3
>1.2 high risk	73	64.6
Spleen size in cm		
01-15 in cm	60	52.6
>15 in cm	54	47.4
Stage of disease		
Chronic	106	93
Accelerated	4	3.5
Blast crisis	4	3.5

5.3. Follow up clinical data

Among 83 CML patients attending follow up starting from one month to twelve years and taking Imatinib 300-800mg PO/day and different TKI drugs during the study period, their WBC median and range were 9.15(2.8-263.90x10^9/L), HGB 13.30(5.6-17.9gm/dL) and PLT 207.50(11.00-1314.00x10^9/L). The clinical data summarized in Table 5 shows that 10.8% had splenomegaly, 3.6% hepatomegaly, 27.7% had disease relapse, 89.2% chronic, 10.8% accelerated phase, and none had blast crisis; Current clinical status is improved in 67.5% worsened in 31.3%, while 61.4% had CHR. Among all the patients 69.9% were BCR-ABL1 positive.

Table 5: Follow-up clinical data of CML patients at Tikur Anbessa Specialized Hospital, Addis Ababa.

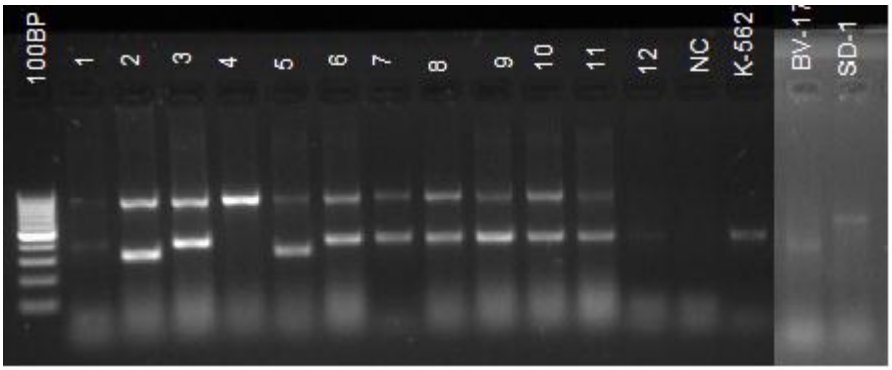
Characteristics	Frequency	Percentage
Splenomegaly		
Yes	9	10.8
No	74	89.2
Hepatomegaly		
Yes	3	3.6
No	80	96.4
Current disease status		
Primary	60	72.3
Relapse	23	27.7
Current disease phase		
Chronic	74	89.2
Accelerated	9	10.8
Blast crisis	0	0
Current clinical status		
Improved	56	67.5
The same	1	1.2
Worsened	26	31.3
Complete hematologic response		
Yes	51	61.4

No	32	38.6
BCR-ABL1		
Positive	58	69.9
Negative	25	31.1

5.4. Patients clinical data across BCR-ABL1 transcript fusion types

Types of BCR-ABL fusion in this study for 31 new cases was b2a2 11(34.5) in 5 males and 6 females. Their median age and hematological characteristic was as follows 34(20-65) in years, WBC 224(35-355.30 $x10^9$/L), HGB 10.9(8.90-12.8gm/dL), PLT 508(120-944 x 10^9/L), b3a2 20(64.5) 11 males and 9 females median and age range was 35(21-50) in years, WBC 230.25(48.20-443.00$x10^9$/L), HGB 9.65 (6.00-13.50gm/dL), PLT 365.50(150-540$x10^9$/L), respectively.

Table 6 shows, you could see the patient clinical database on BCR-ABL transcript fusion types for those who were in treatment TKI and naïf to TKI. There was no significant association between patient's clinical data with BCR-ABL transcript fusion types except for quantitative PCR in which b2a2 fusion types had higher values ranges 55.78(2.15-148.37) than b3a2 35.46(0.01-78.22) and statically significant p-value 0.001.



**100BP- one hundred base pair ladder, sample number 1-12, 4 negatives for bcr-abl, the rest were positive, and NC-negative control, K-562, BV-172, SD-1 were positive control for major and minor transcript fusion types.

Figure 3: Gel-electrophoresis image

Table 6: Patients' clinical data at baseline and follow-up across BCR-ABL1 transcript fusion types.

	%, median, range				
	B2a2	B3a2	B2a2& b3a2	Negative for BCR-ABL	P value
No of case %	32(28.10)	56(49.1)	1(0.9)	25(21.9)	-
The median age in years	34(20-65)	37(20-83)	31	37(19-69)	0.348
Gender (%)					
Male	17(53.1)	38(67.9)	1(100)	17(68)	0.474
Female	15(46.9)	18(32.1)	-	8(32)	
Spleen size (cm) in (%)					
01-15 in cm (%)	18(56.3)	28(50)	1(10)	13(52)	0.746
>15 in cm (%)	14(43.8)	28(50)	-	12(48)	
Median WBC(10^9/L)	221(35-397)	227.40(26-642)	69	266(41-632.55)	0.533
Median HGB (gm/dL)	10.90(6.40-12.90)	9.70(5.7-15.40)	13.6	10.80(7.90-14.60)	0.307
Median PLT (10^9/L)	363(47-1046)	353.50(35-810)	616	333.00(137-1132)	0.533
Blast cell (%)					0.403
01-10 cells	31(96.90)	50(89.30)	1(100)	23(92)	
11-20 cells	1(3.10)	2(3.60)	0	2(8)	
>20 cells	0	4(7.10)	0	0	
Stage of disease (%)					
CP	31(96.90)	51(91.1)	1(100)	23(92)	0.403
AP	1(3.10)	1(1.8)	-	2(8)	

BP	-	4(7.1)	-	-	
Sokal score (%)					
Low	4(12.50)	5(9.09)	0	0	0.243
Intermediate	6(18.8)	15(25.45)	1	10(40)	
High	22(68.8)	36(65.45)	0	15(60)	
Median Transcript load	55.78(2.15-148.37)	35.46(0.01-78.22)		0.0374(0.000-11.30)	0.001

**BCR-ABL negative were patients who were in follows up conditions and BCR-ABL was below the detection limit

5.5. Follow up clinical data across BCR-ABL transcript fusion types

From follow-up patients who were taking first, second and third generations of TKI their follow-up clinical data versus types of bcr-abl fusion genes were seen. When b2a2 21(25.3), b3a2 36(43.4), b2a2&b3a3 1 co-expressed (1.2), and the rest were negative for BCR-ABL 25 (30.1). The patient's clinical data across BCR-ABL was assessed to know whether there was an association between clinical data and bcr-abl transcript types. There was no significant association between age, gender, disease stage, others, and hematologic parameters except for WBC (p=0.001) and HGB (p=0.003), current disease status relative to the primary where relapse was higher b3a2 15(41.7) and b2a2 7(33.3) (p-value =0.011). Also, the duration of TKI versus types transcript seen was higher in patients taking TKI drug for more than 12 months especially for b3a2 26(72.2) and bcr-abl negative patients were also taking the TKI drug for more than 12 months 24(96); their follow-up ranges 60 (9-144) month, p=0.005.BCR-ABL negative patients had more complete hematologic responses than others p, (0.0001). No molecular response was more common in both transcript types and the deep molecular response was noted in BCR-ABL negative patients when the p-value was 0.0001. Quantitative transcript load (IS %) had a significant association with BRC-ABL fusion types; in which b2a2 has a high amount transcript load 55.78(0.13-75.68), followed by b3a2 34.85(0.01-96.87), while bcr-abl negative had 0.0374(0.00-11.30) p-value 0.001, and others details are shown in Table 7, Figures 5 and 6.

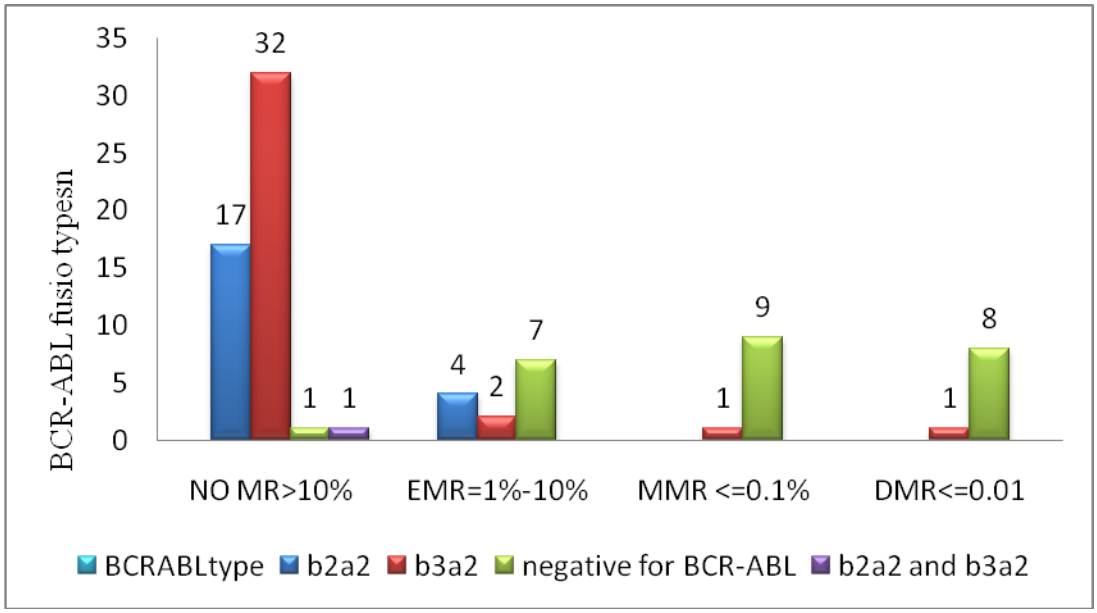
Table 7: Follow up clinical data across BCR-ABL transcript fusion types

	%, median, and range

	B2a2	B3a2	B2a2& B3a2	Negative for BCR-ABL	P- value
No of case %	21(25.3)	36(43.4)	1(1.2)	25(30.1)	-
The median age	32(22-60)	37.5(20-83)	31	37.5(19-69)	0.135
Gender (%)					
Male	12(57.1)	27(75)	1	17(64)	0.463
Female	9(42.9)	9(25)	-	8(36)	
Median WBC(10^9/L)	17.9(3.2-263.9)	15.05(2.80-180)	4.2	5.8(3-18.9)	0.001
Median HGB(gm/dL)	12.8(9.1-16.9)	12.80(5.6-17.9)	13.1	14.2(9-17)	0.003
Median PLT (10^9/L)	246(11-1205)	179.5(13-1314)	74	208(88-390)	0.359
Splenomegaly (%)					0.078
Yes	5(23.8)	4(11.1)		-	
No	16(76.2)	32(97.2)	1(100)	25(100)	
Hepatomegaly(%)					
Yes	2(9.5)	1(2.8)		-	
No	19(90.5)	35(97.2)	1(100)	25(100)	0.369
Current diseases status(%)					
Primary	14(66.7)	21(58.3)		24(96)	0.011
Relapse	7(33.3)	15(41.7)		1(4)	
Stage of disease (%)					
CP	18(85.7)	30(83.3)	1(100)	25(100)	0.197
AP	3(14.3)	6(16.7)	-		
Currentclinical status(%)					
Improved	13(61.9)	18(50)	1	24(96)	0.286
The same	1(4.8)				
Worsened	7(33.3)	18(50)		1(4)	
Regime or dosage					0.165

change history(%)					
Yes	2(9.5)	11(30.6)		3(12)	
No	19(90.5)	25(69.4)	1	22(88)	
Treatment discontinued history (%)					0.088
Yes	9(42.8)	10(27.8)		3(12)	
No	12(57.2)	26(72.2)		22(88)	
Type of TKI (%)					
Imatinib 300mg	0	0	0	1(4)	0.889
Imatinib 400mg	19(90.5)	26(72.2)	1(100)	21(84)	
Imatinib 600mg	0	1(2.8)	0	0	
Nilotinib	2(9.5)	7(19.4)	0	2(8)	
Dasatinib	0	0	0	1(4)	
Bosutinib	0	1(2.8)	0	0	
Ponatinib		1(2.8)			
CHR					0.0001
Yes	9(42.9)	17(47.2)		24(96)	
No	12(57.1)	19(52.8)		1(4)	
Median follow up in month	36(1-132)	31.5(1-144)	11	60(9-144)	0.0001
Median Transcript load (IS %)	55.78(0.13-75.68))	34.85(0.01-96.87)	21.0573	0.0374(0.00-11.30)	0.001

** Negative for BCR-ABL means it is below detection limit by multiplex PCR.



** NMR-no molecular response, EMT-early molecular response, MMR-major molecular response, DMR-deep molecular response P-value 0.0001.Negative BCR-ABL-'below detection limit.

Figure 4: BCR-ABL fusion type's versus Molecular Response in IS%

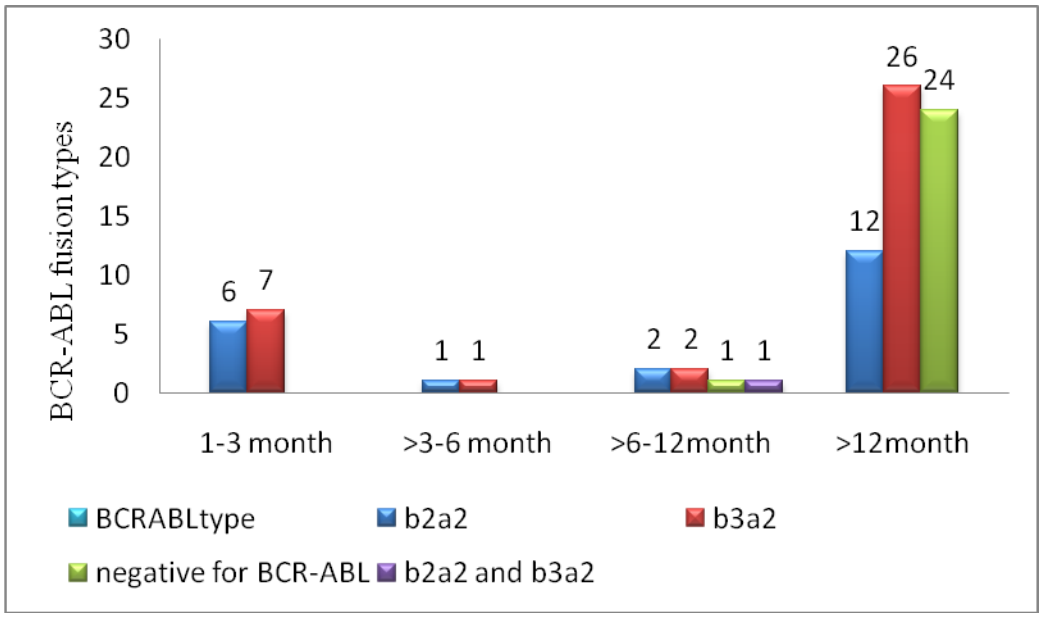


Figure 5.The BCR-ABL1fusiontypes in Y-axis versus duration of treatments in month interval in the X-axis.

5.6. Comparison of multiplex RT-PCR with the quantitative multiplex RT-PCR for chronic myeloid leukemia patients

To test the specificity and sensitivity of multiplex RT-PCR assay and decide the cut-off values by ROC curve by comparing against dual multiplex RT-qPCR, the detection of fusion genes was first determined using agarose gel by preparing the master mix and primer needed for the qualitative examination. The fusion genes were obtained by extracting RNA from lysed white blood cells then synthesizing cDNA for multiplex PCR to run. The BCR-ABL fusion gene types were compared against the control genes to know the types of their translocations whether B2a2 or B3a2 meaning BCR-ABL positive or not. The result showed 31 b2a2, 56 b3a2, b2a2&b3a2, and 25 samples were negative for BCR-ABL fusion genes by this assay. After knowing their BCR-ABL status for 114 patients, all samples were tested by dual multiplex RT-q PCR two times. The BCR-ABL and ABL1 were amplified from the same cDNA. Quantification was done using the standard curves BCR-ABL and ABL ranges from 10^6 to 10^1). The amount of BCR-ABL transcripts load in patients was further normalized to the reference gene ABL1 as the normalized copy number (NCN). The results showed that the percentage of the M- BCR-ABL copies% to ABL copies % time's conversion factors settled for our setting 0.542. Means, M-BCR-ABL (copies /2 µl cDNA), ABL (copies /2 µl cDNA), sum-BCR-ABL, sum ABL, copies M-BCR-ABL/ABL [%], IS% times (%conversion factor settled for study, 0,542). We got results ranges from 0.0001 IS to 148.37.

Of tested 114 CML patients by multiplex RT-PCR and quantitative multiplex RT-PCR to determine the cut-off value for multiplex reverse transcriptase relative to dual quantitative multiplex RT- PCR. Cutoff value was determined at area under the curve 0.977(97.7 %), SE values 0.013(1.3%), CI 95% (0.952 to 1, P-value 0.0001) was IS % 0.31. A sensitivity 96.6%, and 1-specificity of 0.88(88%).This means the multiplex reverse transcriptase conventional method truly identified positively as positive at 96.6% and negative as negative at0.88(88%) giving the sensitivity and specificity, respectively. A positive and negative predictive value was also calculated to further validate the cut-off values. The result was the same with sensitivity and specificity. Standard curve for BCR- ABL and ABL in Figure 6: Plasmid BCR-ABL1 and ABL1 copy number-based standard curves.

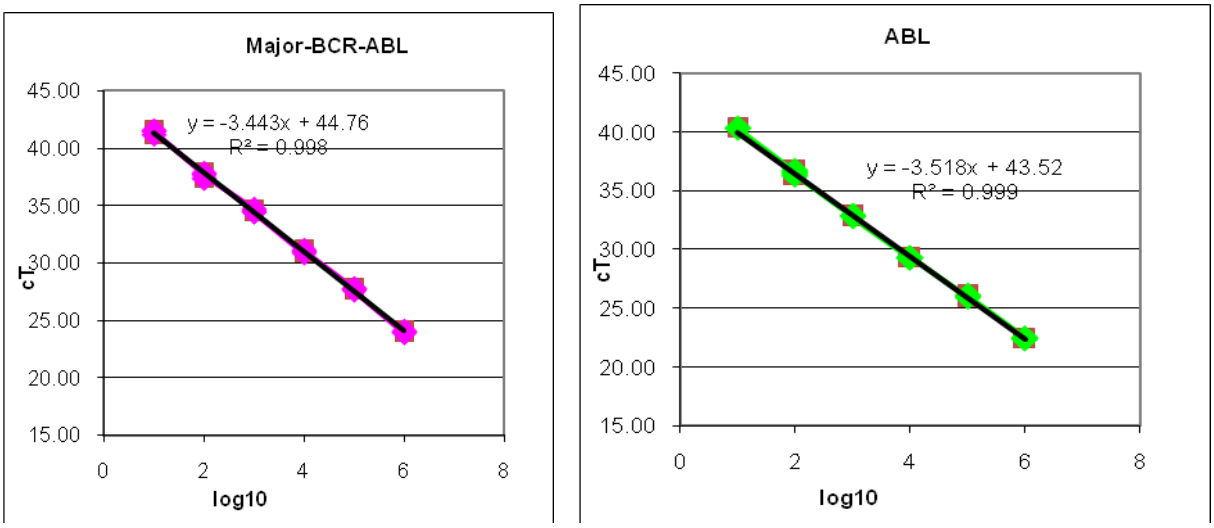


Figure 6: Plasmid BCR-ABL1 and ABL1 copy number-based standard curves.

Table 8: Test accuracy of multiplex RT-PCR relative Quantitative PCR

Truth (Reference method quantitative PCR)			
		+ (>= 0.31%)IS	- (< 0.31%) IS
Test(M-PCR)	Test positive	86	3
		True positive	False-positive
	Test negative	3	22
		False-negative	True negative

`Summary indices of test performance

TPF= true positive fractions (sensitivity) =TP/(TP+FN)= 86/(86+3)= 96.62

FNF=False negative fractions (1-sensitivity) = FN/ (TP+FN) = 3/ (22+3) = 0.12

TNF=True negative fraction (specificity) = TN/ (TN+FP) = 22/ (22+3) = 0.88

FPF=False positive fraction (1-specificity) = FP/ (TN+FP) = 3/ (22+3) = 0.12

PPV=Positive predicted value=TP/ (TP+FP) = 86/ (86+3) = 0.9662

NPV= Negative predicted value = TN / (TN+FN) = 22(22+3) = 0.88

In addition, the roc curve was calculated for b2a2 and b3a2 to see the difference between them in terms of sensitivity and specificity. For b2a2, the cutoff value was determined at 2. 15% IS, at the area under the curve 0.986(98.6%), standard error 0.012(1.2%) and CI 95% (0.963 to 1) P value were 0.0001. The sensitivity and specificity of b2a2 for multiplex PCR were 96.9% and 96% respectively at CI 95% had a false-negative rate was 4%. Again for b3a2, the cutoff value was determined at 0.31 which was similar to the general one at the area under the curve 0.971(97.1%), standard errors 0.019(1.9%), and CI 95 % (0.935 lower limits to 1 upper limit)

P-value was 0.0001Sensetivity and specificity of B3a2 for multiplex PCR was 96.4% and 88% and had false-negative rate 12%. The results are shown in Figures 8 to 10.

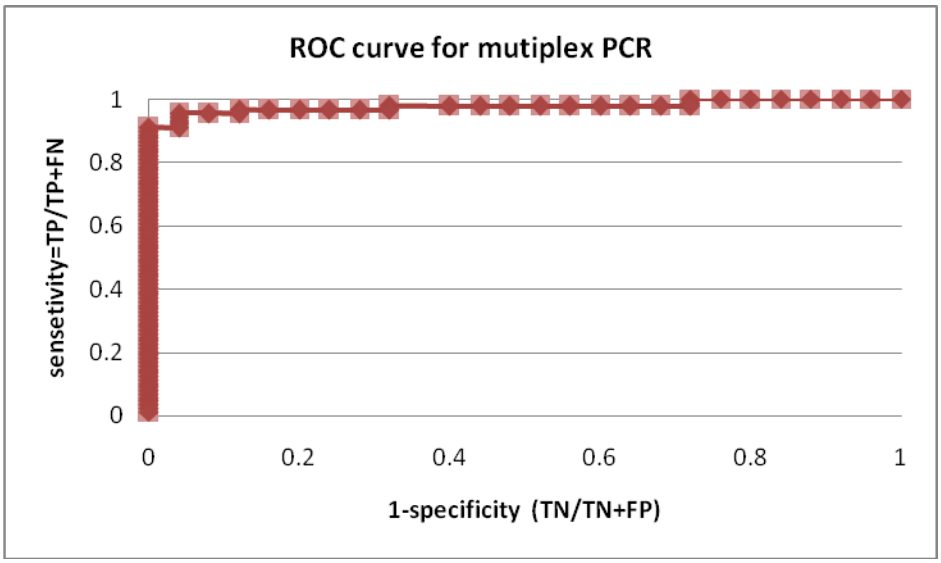
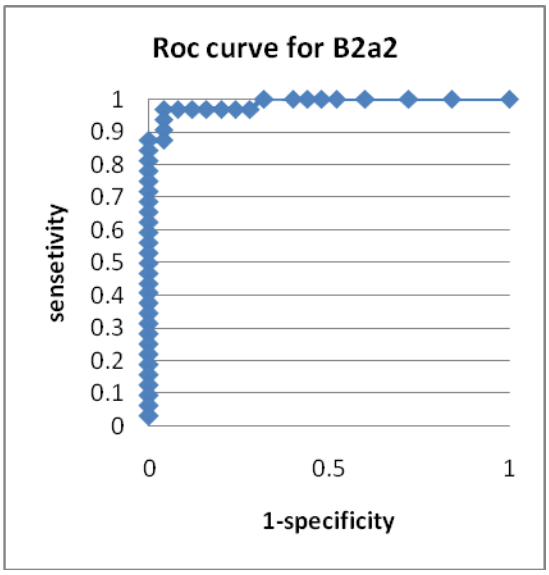


Figure 7: ROC curve for multiplex RT-PCR for chronic myeloid leukemia



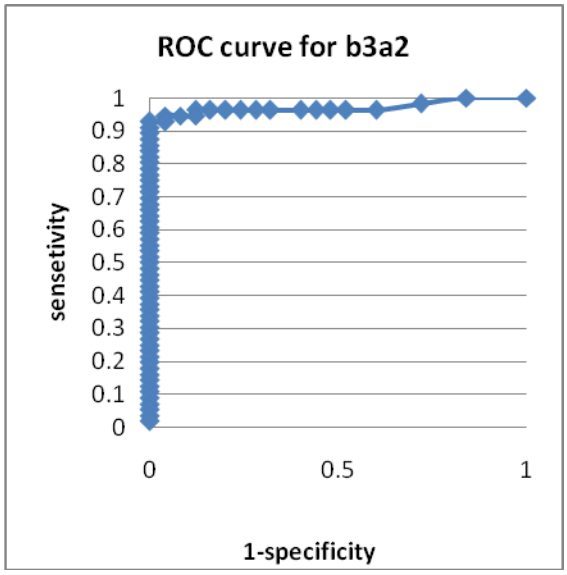


Figure 8: A, Roc curve for B2a2 B, Roc curve for B3a2

6. Discussion

Tikur Anbessa Specialized hospital was the first referral hospital and center for cancer patient treatments in our country including CML; others blood cancers, and solid cancers. The main objective of this study was to evaluate Multiplex Polymerase Chain Reaction (PCR) as Prognostic Tools for chronic myeloid Leukemia (CML) Patients in Resource-Limited Setting at TASH with a comparison with dual quantitative –PCR. This is because, our patient's sample still sent abroad for confirmation of diagnosis and treatment follow-up conditions, which was paid by patients own pocket money which is expensive for them, even most of them could not afford the cost for doing so, that was the reason this study was initiated to evaluate this method as an alternative with low cost relative to others.

In this study, 114 patients enrolled including 31patients who were naïve to treatment and 83 patients were in follow-up clinic. Of the total,72 were males and the median age for both was 35(19-83) which was nearly similar with Indian patients whose median age was 39(years)and northern east Iran Patients' whose age range 21 to 77 years with a mean and standard deviation of45.89±15.60 years(48, 56).In the current study, the age range for most patients was similar to reports for Asians 47.5%(20-39), and African countries 43.4%(20-39). But the age group in the current study was lower than Europeans 48.5%(40-64), and Latin American42.8%(40-64)(57). The study shows younger productive age was at higher risk than others, which still need long mile journey to come across the reason behind why Ethiopian CML patients are younger.

From the study participants, 112(98.30) had developed splenomegaly and 57 (49.10) had developed hepatomegaly at baseline. Their spleen size median in cm was 14.75(4-30) and. The blast count median was 4(0-45) percent, the Sokal score for low risk was 9(7.8), intermediate-risk 32(27.6), and high risk 73(64.6). The median and range for WBC were $229.93x10^9$/L (26-642), PLT $344.50x10^9$/L (35-1132), and HGB gm/dL10.30 (5.7-15.4) at the baseline for both new case and follow-up patients.

Baseline clinical data were assessed across BCR-ABL transcript fusion types for both new cases and follow-up patients, including those who were negative for multiplex PCR during follow-up, were also included in this study. The BCR-ABL fusion genes types for b2a2 was 32(28.1), b3a2 56(49.1), b2a2&b3a2 co-expressed 1(0.9) and negative (undetectable) for BCR-ABL fusion genes was 25(21.9). But, for the 31 new cases in the current study, their frequency for b2a2 was 11(34.5%) and b3a2 20(64.5%).

In the current study new cases and follow patients had more b3a2 frequency relative to the others across the study participants. That was similar to a study conducted in Indian where the frequencies reported were b2a2 36.36%, b3a2 63.53%, and b2a2 2.94 % (58). Another study shows occurrence of b2a2, b3a2, and b2a2 &b3a2 transcripts indicated as frequencies 28.84%, 66.82%, and 3.36%, respectively (59). Also, another study reported the frequencies of b2a2 and b3a2 to be 32% and 68%, respectively (60). In the current study, there was no significant association between transcript types with age and gender. However, those with b2a2 were younger 34(20-65) than 3a2 37(20-83) similar to a study done in Syrian patients with b3a2 transcript were younger than patients with b2a2 transcript (61).

Male patients had more frequent b3a2 (67.9) than females 18(32.1) in this study a finding which is in concordance with other studies that showed males had more (60). On the other hand, others done in Sudan reported that males had more b2a2, and females had more b3a2 (62). This study did not indicate any significant transcript association with baseline blast cells count percentage, spleen size, Sokal score, and stage of disease in which similar to a study conducted by others (4). There was no significant association of hematological parameters with BCR-ABL fusion types except, quantitative PCR shows significant association with BCR-ABL in which b2a2 fusion types had higher values ranges 55.78(2.15-148.37) than b3a2 35.46(0.01-78.22) and statically significant p-value 0.001. White blood cell counts lowered in b2a2, 221(35-397) than b3a2 had more 227 $x10^9$/L (26-642), and platelet count was higher in b2a2 than b3a2 ranges $363x10^9$/L (47-1046), and $353.50x10^9$/L(35-810) respectively p-value was 0.533. It was similar to the study conducted in Iran where patients with b2a2 had WBC $200x10^9$/L(17-800), b3a2 was $215x10^9$/L(21-775),(63). Another study reveals that platelet count was higher in patients holding b3a2 (4), which was inconsistent with the current finding, where patients withb2a2 had a platelet count higher $370x10^9$/L(47-1046) than b3a2,353.50 x 10^9/L(35-810) which was also similar with the study conducted in Syrian where the platelet count was higher in patients expressing b2a2 mean was 293 x 10^9/L than those with b3a2 transcript mean was 257 x 10^9/L (61).

Patients who were in follow-up conditions and taking different TKI from one month to one hundred forty months were assessed for their clinical data with their bcr-abl transcripts. A higher frequency of bcr-abl expression was seen for b3a2 patients than b2a2 patients. This result was similar to the study done in India(64), which shows b3a2 was higher in their patients. For most, there was no significant association between clinical data and bcr-abl expression in the following situations except for some of them. HGB was lowered in b3a2 12.8gm/dL(5.6-17.9)

than b2a2 12.8(9.1-16.9), P-value=0.003), current disease status relative to the primary where relapse was higher b3a2 15(41.7) and b2a2 7(33.3) (p-value=0.011) also, duration of TKI p, (0.0001)this was more common in patients taking the drug for more than 12 months which indicates there was treatment failure for bcr-abl, positive patients, at most. Quantitative transcript load (IS %) had significant association with BRC-ABL fusion types; in which b2a2 has high amount transcript load 55.78(0.13-75.68), followed by b3a2 34.85(0.01-96.87), while bcr-abl negative had 0.0374(0.00-11.30) p-value 0.001. No molecular response was more common in both transcript types and the deep molecular response was noted in BCR-ABL negative. No significant association between molecular responses with the duration of treatment was seen, p-value 0.097 in which, most of the follow-up patients were positive for bcr-abl1genes.

Several years back many countries establish qualitative, real-time, quantitative, and recently digital drop PCR assays to measure the bcr- abl transcript load in the peripheral blood, and bone marrow to diagnose and monitoring of molecular response against their treatment response(65-67). The transcript load indicates the amount of oncogene presented in blood or bone marrow in diagnosis or follow-up conditions(68).

For patients on follow-up, the European Leukemia Net suggested for treating the bcr-abl transcript level for MMR < 0.1%, MMR4< 0.01%, MR4.5<0.0032%, MR5<0.0001% the least criteria required for quantifications. For treating the CML expressed as bcr-abl the milestone need in IS was patients have the optimal response at 3 months ≤10%, warning>10%, failure>10%, if confirmed within 1-3 months, 6 months ≤1% optimal response, warning1-10%, failure >10%, while at 12 months ≤0.1 optimal response,0.1-1% warning, >1% failure and any times ≤0.1% optimal response, >0.1–1% & loss of ≤0.1% (MMR)a warning, and>1%, resistance mutations failure, high-risk ACA. For patients aiming at TFR, the optimal response (at any time) is BCR-ABL1 ≤ 0.01% (MR4). A change of treatment may be considered if MMR is not reached by 36–48 months(69). This was not done in our country due to resource limitations.

The current study was designed with the ultimate goal of applying the multiplex RT-PCR method as a prognostic tool in TASH for follow-up patients. The method was compared with the golden standard quantitative RT-PCR, and the cut-off value for multiplex RT- PCR was determined to explore further regarding the method's potential to be applied for prognostic tools for CML patients. This is because there was no molecular response measurement assay in our country for patients taking TKI for their treatments due to several resources issue. The prognostic utility was assessed across the patient's follow-up clinical data with bcr-abl

expression level in terms of positivity and negativity for those taking the drug from one month to 12 years. All our new cases and follow-up patients who were positive with the multiplex RT-PCR showed more than 95% agreement with quantitative PCR. Which was similar to a study conducted on Indian patients with 100 % agreement between them (66). There were 25 (30.1) PCR negative patients, with the median duration for achieving the multiplex PCR negativity was 60(9-144) months. The method was evaluated in terms of sensitivity; specificity, and by performing a ROC curve for knowing the limit of detections relative to the golden standard multiple RT q-PCR which is mostly used as prognostic tools in developing countries.

This study evaluated by comparing the bcr-abl expression in terms of negative if the BCR band was clearly visualized and positive if both BCR-ABL bands were seen, and BCR band was also visualized as control at most in the gel- electrophoresis relative to the golden standard quantitative PCR. To determine, the limit of detection for multiplex RT- PCR by calculating cut-off value in ROC curve in excels 2007 Microsoft ware comparing with reference method quantitative PCR. First, the area under the curve was calculated at CI 95 (ranges from 0.952 to 1) with a P-value (0.0001), a standard error of 0.013(1.3%), and AUC was 0.977(97.7%) then, the cutoff values were determined for multiplex RT-PCT at 0.31 IS % transcript load that was measured by quantitative multiplex RT- PCR. These cutoff values were determined at sensitivity 0.9662(96.62) % and specificity 0.88(88%). In this study, multiplex PCR detected bcr-abl transcript load to the level equal to or greater than IS 0.31%, which means, the method could detect results above or equal to 0.31% IS positive as positive 96.62% negative as negative 88 % at CI 95. False negativity of the method was 12% at CI 95. However, it reveals very good performance because the area under the curve was 97.7% which mostly recommended for method evaluation was a maximum 1 minimum 0.5 (70).

In evaluating the multiplex PCR to know the limit of detection for major types transcript b2a2 and b3a2. ROC curve was calculated for both. The multiplex PCR was sensitive and specific for b2a2 96.9% and 96 respectively at the cutoff value 2.15 % IS when the area under the curve was 98.6% and P-value was 0.0001, while for b3a2 multiplex, PCR was sensitive and specific 96.4% and 88% respectively at the cutoff value 0.31 % IS when the area under the curve was 97.1% and P-value 0.0001. Multiplex PCR sensitive for both types of the transcript above 95% but the specificity of multiplex PCR was more specific to b2a2 at 96% than b3a2 88%. But, depending on the limit of detection for both in the number of transcripts copy our multiplex PCR detected as lower as 0.31% IS for b3a2 at 96.4%& 88% sensitivity and specificity respectively.

Whereas in the case of B2a2 its detection limit was as lower as 2.15 % IS at 96.9&96 sensitivity and specificity respectively. That means the current method was not different in terms of sensitivity for both transcripts but more specific to detect b2a2 than b3a2. A comparative study conducted in South Korea shows both transcripts were sensitive for multiplex PCR (49).

An effort was made to compare the multiplex RT-PCR method detection limit with other studies around the world but no similar published study was found because they evaluated most of their method relative to two other methods which we could not do it so. However, it was tried to assess our method by selecting from them relatively concordance with us. The multiplex PCR method applied in the present study was a quick and realistic qualitative assay that used four forward and four reverse primaries, for major and minor BCR-ABL transcripts fusion types. Its sensitivity ranges from 0.952 to 1 at 95% CI relative to quantitative RT-PCR. Therefore, multiplex RT-PCR could detect one Philadelphia chromosome-positive from 100 normal cells in one microgram of RNA. The study conducted in Tel Aviv Israel shows something similar with us in terms of sensitivity that was 1: 1000 for multiplex RT-PCR and suggested that sensitivity at this level was important for confirmations clinical disease and or preclinical relapse a little bit higher than FISH in their setup(71).

The quantitative PCR done in the current study for both new and case follow-up the median transcript load was 34.30(0.0001-148.37). In follow-up cases for 58(69.9%) positive and 25(30.1%) negative the transcript load median was 37.23(0.1-78.22), and 0.0374(0.0001-11.3) respectively. From 25 (30.1)multiplex RT-PCR bcr-abl negative patients false positive at our cut-off value was 3 whereas 22 were true negative and true positive was 86 and false-positive were 3. Of the one hundred fourteen quantitative PCR tests, 86(75.4%) had positive BCR-ABL/ABL result greater or equal to 0.31%and 22(19.7%) had negative BCR-ABL/ABL result in less than 0.31 % IS. This was similar to again study done in Tel Aviv Israel where 81% of all patients with negative multiplex RT-PCR results assessed by RQ-PCR had a BCR–ABL/ABL ratio below or equal to 2% and 6of the 32 patients (19%) had a BCR–ABL/ ABL ratio above 2% (71). This result was also indicated for patients having BCR-ABL/ABL transcript load less than 0.31 % IS were mostly in major molecular response relative to the European leukemia net recommend for the major molecular response was less than 0.1% in IS (69).

Therefore, this qualitative multiplex RT-PCR was practical for the detection of BCR-ABL fusion genes transcript for the majors and minors with higher sensitivity and specificity. It

will support the clinical diagnosis and prognostic values depending on the presence or absence of the band which is indicative of as the patients were in major molecular response or not.

7. Strength and limitation of the study

7.1. Strength of the Study

- Data collection was done from both the medical record and I care system which enables getting the full picture of the patient's clinical status and current conditions they were dealing with.
- The study was done with close supervision of seniors' hematology sub-specialties doctors and senior hematology nurses to access patients' data in the full package and explaining our main objective to them to have a clear idea about this study.
- The study also facilitates the learning of different molecular skills and for potential application in our premier tertiary care hospital to improve CML patient's management.

7.2. Limitation of the Study.

- A key limitation of this method was only evaluated relative to the gold standard quantitative RT-PCR, but not other methods to further evaluate more. However, the study shades light that the multiplex PCR can be applied in our tertiary care hospitals for managing CML patients.

8. Conclusion and Recommendations

In conclusion, the patient clinical data at baseline and follow-up had no significant association with bcr-abl transcript types except quantitative PCR transcript load in both baseline and follow-up conditions. In follow-up patients, clinical data had a significant association with a type of bcr-abl transcript for WBC, complete hematologic response, molecular response, and duration of treatment, quantitative PCR transcript load and patient's disease status. Evaluations of multiplex PCR as prognostic tools for CML patients in follow-up conditions revealed that its detection limit goes up to 0.31% IS at sensitivity 0.9662(96.62%) and specificity 0.88(88%) which, mostly indicate the patients are in major molecular response for the treatments they are taking.

Based on the findings, it is recommended if Tikur Anbessa Specialized Hospital used this finding for CML patients as diagnostic and prognostic tools with other clinical parameters to treat the patients, and for knowing their molecular response against the treatment they are taking. The multiplex PCR is relatively cheap and accessible than other methods that are used in monitoring molecular response for CML patients which was expensive to perform. Therefore, the final recommendation for the Ethiopian Ministry of Health and other stakeholders who are concerned for health should have to give attention to chronic myeloid leukemia patients by assisting the implementation of such molecular patients at least in the tertiary care hospitals managing CML patients. Because our physicians are treating their patients, without knowing their molecular response for the treatment they are talking about, unlike most countries around the world which used molecular response as a monitoring mechanism.

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7. References

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9. Annex

Annex I: standard operating procedures protocol

Reagents and materials needed for Red blood cell lysis

- Ammonium chloride (NH4Clx1),
- Phosphate buffer saline (PBS);
- Guanine thiocyanate(GTC) with β -mercaptoethanol;
- Centrifuge (capable of spinning volumes of up to 40 mL);
- Syringes (20 mL) (from BD plasticware);
- 18G Blunt end mixing needles.
- Ice
- Pasteur pipette
- Microscope
- hemocytometric chamber to count WBC
- Trypan blue

SOP forNHCLX8 and GTC preparations

- The dilution is De-ionized water
- Weigh the following powders first! (put in Aluminum foil). 33.2g NH_4Cl,
  0.1488g EDTA
  4g $KHCO_3$
- Put all the above powders into a 500 ml Erlenmeyer flask, and add water up to 500 marks! Add water little by little until completely dissolved.
- This means it is 8 X concentration. Because under normal conditions you were supposed to dilute it up 4000ml. (4000/500 = 8)
- The reason I prepare in 8x concentration is to keep the solution safe from contamination.
- Keep in ice
- Next time if you want to prepare a working solution, take 100ml of the concentrate (8x) and add 700ml water. i.ea total of 800ml with (1x).
- GTC-Buffer (10ml 0.5M Sodium Citrate, pH7 + 1g SARCOSYL + 94.52g GTC fill up to 200ml with DEPC-H_2O); 4M GTC,25mM Sodium citrate (pH 7),0.5% Sarkosyl), for use solution : + 8microliter/ml β-Mercaptoethanol, Solution is stable for 4 weeks. Keep in ice

Protocol

Sample preparation and RLT cell lysis.

Note: all possible steps should ideally be performed within a Class II cabinet. Use aseptic techniques throughout and make sure the tubes are firmly capped. Reagents should be stored in small aliquots.

1 Transfer whole sample (BM, PB, and bone marrow harvests) to 50 mL suitably labeled polypropylene (Falcon) tubes. The tube should carry at least two identifiers for the patient (Surname and laboratory number)

Erythrocyte Lysis Protocol

1) Sample type: Whole blood, collected using EDTA/Heparin tube
2) Centrifuge the whole blood at 1500 rpm for 5 minutes
3) Using a plastic paster pipette, aspirate and discard the supernatant until it reached 0.5 cm above the buffy coat layer.
4) Transfer the whole buffy-coat layer into to 50 ml falcon tube. Make sure to include RBC at the bottom of the buffy coat so that the WBC component would be harvested.
5) Add the NH_4Cl 1 x upto40ml of falcon tubes and mix well (by inverting the tube 8x!).
6) Put in ice for 30 minutes and mix intermittently

a) In the meantime please don't forget to put your PBS solution in ice-cold, so that it won't shock the cells during the next step.

b) Check the RBC for complete lysis of RBC, if so next to the next step

7) Centrifuge for 5minutes at 1500rpm at 4^0C(it is critical because the cells were in ice, the temperature of your centrifuge should be ready at 4^0C, beforehand!)
8) Aspirate the supernatant using a paster pipette, because the pellet is loos, if you decant the tube, some WBC could be washed off! Make sure to leave enough fluid with the pellet at the bottom
9) Check the pellet if there are still unlysed RBC, if there is much, then

a) Add again NH_4Cl (1x) like 10 ml, and incubate for additional 10-15 minutes, in ice. (But make sure the solution is cold!),

b) Then Aspirate the supernatant using a paster pipette, proceed to the next step

10) Vortex the pellet and Ice cold PBS up to 30 ml for washing off lysed RBCs.
11) Centrifuge at 1500rpm for 5 minutes and discard the supernatant again
12) Vortex briefly to avoid clumps of cells, and add 30 ml ice-cold PBS,

13) Centrifuge at 1500rpm for 5 minutes and discard the supernatant leaving behind approximately 2ml, to help you count the cells!

a) Vortex. And count the cells, Calculate your total cell count = cells/ml

14) Then you can aliquot a maximum of 10 X 10^7 cells in each nun tubes ('A' & 'B').

a) Centrifuge the two Nunc tubes for 3min/1300rpm/RT, discard the sups, and blot completely on tissue paper.

15) Add 600 µl GTC (with betamercapto ethanol) into each of the Nunc tubes.13. Mix several times until the pellet homogenize

14. Store the GTC lysate at -80.

Total RNA Extraction (RNeasy Mini Kit) Optional:co-extraction of genomic DNA& Protein.

Adopted from Leipzig University, Hematology oncology LAB, by Samuel K, "Genotyping of CML" Oct 19,2018

1. Sample type:

❖ IF: Whole blood, Bone Marrow EDTA/Heparin

- Lysed, washed pellet in 1-2ml PBS, re-suspend
- Adjust max. cells 5 X 10^6/200 microliter or [2.5 x 10^6/ml]

❖ IF: GTC, 200microliter GTC Lysate in 1.5 ml PBS

2. Materials and Equipment

❖ RNeasy Mini Kit (QIAGEN 74106)

❖ 100% Ethanol (AppliChem, A3678,1000), 70% Ethanol

❖ RNase-Free DNase Set (50) (QIAGEN 79254)

❖ QIAshredder (250)(QIAGEN 79656)

❖ Thermoblock, Nanophotometer (260,280nm), Bench Centrifuge

3. Frozen lysates should be completely thawed and salts are dissolved.

❖ Avoid prolonged incubation, which may compromise RNA integrity. Optional: If any insoluble material is visible, centrifuge for 5 min at 3000–5000 x g. Transfer supernatant to a new RNase-free glass or polypropylene tube,

❖ Thoroughly vortex until the solution is homogenous/slimy, and continue with step 4

4. Pipet the lysate (Max, 600 µl, at a time) directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at full speed ≥8000 Xg (≥10,000rpm)

5. Add 600µl (or 1 volume of flow-through homogenate) 70% ethanol and mix well by pipetting.

6. Transfer up to 600 µl (possible upto700 µl) of the ethanol vs sample mix, to an RNeasy spin column placed in a 2 ml collection tube.
7. Centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through Or keep it (Optional).
8. Transfer the remaining flow-through homogenate into the same RNeasy spin column.
9. Centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through.
10. Add 350 µl Buffer RW1 to the RNeasy spin column (washing step), close the lid.
11. Centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm)&discard flow-through, re-use collection tube next step
12. Add 80 µlDNase I directly to the RNeasy spin column membrane, and put at RTM for 25 min.

❖ Preparation DNAse I: Add 10 µlDNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube, and (optional) centrifuge briefly to collect residual liquid from the sides of the tube.

❖ Mixing by gently inverting the tube. Do not vortex! It is stable for six weeks at 2-8^{0}C.

13. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently,
14. Centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through
15. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently.

❖ Ensure that ethanol is added to Buffer RPE before use,

16. Centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) (to wash the membrane), discard flow-through.
17. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently.
18. Centrifuge for 2min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane.

❖ Ensuring no carry-over of ethanol during RNA elution. May interfere with downstream reactions.

19. <u>Optional:</u> Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently.
20. Centrifuge at full speed for 1 min.

❖ Place the RNeasy spin column in a new 1.5 ml collection tube (supplied).

❖ Elution: Add 40 µlRNase-free water directly to the spin column membrane.

21. Close the lid gently, and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.
22. If the expected RNA yield is >30 µg, repeat using another 30–50 µlRNasefree water, or using the eluate from the last step 10 (if high RNA concentration is required).

Reagent and material used for cDNA synthesis

- Superscript IV VILO Mastermix, Thermo Fisher Scientific # 11756500(500 reaction)

- PCR tube
- Thermocycler
- RNA

Noted: Some laboratories use the entire elute from 1/3 of the original RLT or GTC lysed samples; others use a specified amount of RNA (after OD reading).

The protocol for synthesizing cDNA by using superscript IV VILO Mastermix, Thermo Fisher Scientific # 11756500(500 reaction) were

- add 8µl of superscript with 32µl of RNA extracted a total volume of 40µl then incubate it in thermocycler as protocol written here

Thermo cycler protocol for cDNA Synthesis

- 10 minutes for 25 C^o (annealing primers)
- 10 minutes for 50 C^o(reverse transcribe RNA)
- 5 minutes for 85 C^o (inactivate enzyme)
- 12 C^o forever
- Store cDNA samples at - 80^{o}C.

cDNA master mix preparation

A master mix should be prepared to achieve the following recommended working concentrations in each reaction:

Working concentration

Working concentration	
5xbuffer*	x reaction
0.1 mol/L DTT	10 mmol/L
dNTP 25 mmol/L	0.5 mmol/L
Random primers	500 ng
RNasin	50 units
Reverse transcriptase	400 units
ddH2O Volume will vary depending on final volume reaction and volume of RNA used	The volume will vary depending on final volume reaction and volume of RNA used

Multiplex BCR-ABL PCR

1. Definition

- ❖ BCR - breakpoint cluster region
- ❖ ABL - Abelson murine leukemia viral oncogene homolog
- ❖ M - Major
- ❖ m - minor
- ❖ RT – Reverse Transcriptase

2. Materials and Equipment

- ❖ PCR Cycler
- ❖ Agarose (Serva, 11404)
- ❖ GelRed (VWR, 730-2958DE)
- ❖ Taq-Polymerase (Applied Biosystems, N8080153)
- ❖ cDNA from cell lines; K562, SD-1, BV173
- ❖ Bench Centrifuge, Microcentrifuge
- ❖ Buffer (1ml Bromophenol blue [0,25%] in Ficoll-solution [25%] + 9ml Sucrose-solution [40%])
- ❖ Gel electrophoresis set, Gel camera

3.*Primer 5´→3´:*

- ❖ BCR F3: ACCGCATGTTCCGGGACAAAAG (BCR Exon 1)
- ❖ BCR F4: ACAGaATTCCGCTGACCATCAATAAG (BCR Exon 13)
- ❖ BCR R1: ataggaTCCTTTGCAACCGGGTCTGAA (BCR Exon 21)
- ❖ ABL R2: TGTTGACTGGCGTGATGTAGTTGCTTGG (ABL Exon 3)

4.Procedure

- ❖ Sample: Starting material is cDNA from BCR-ABL positive CML patients
- ❖ BCR-ABL PCR master mix should be pipetted in the cleanroom (Room #?) BCR-ABL PCR master mixes are aliquoted at -80 ° C frozen
- ❖ Template (cDNA) should be pipetted in-room #?
- ❖ Pipette 23 µl per reaction volume
- ❖ 2,3µl 10x Buffer
- ❖ 2.3µl MgCl2 [25mM]
- ❖ 0.184µl dNTPs [25mM]
- ❖ 0.46µl primer mix (BCR R1, BCR F3, BCR F4, ABL R2 [12.5µM each])
- ❖ 15.656µl of water

Total of 20,9µl~=21 µl

- Add 0.1 µlTaq polymerase [5units / µl] and 2 µlcDNA per sample (in room temperature)
- Add controls; to minimize contamination, better controls be added before the sample templates
- Positive controls (after transcription of RNA ~ 0.1 µg / µl)
- cDNA of K562 cells as M-BCR-ABL controls (b3/a2) 385bp
- cDNA of BV173 cells as M-BCR-ABL controls (b2/a2) 310bp
- cDNA of SD-1 cells as m-BCR-ABL control (e1/a2) 481bp
- Negative controls:
- NTC (multiplex BCR-ABL PCR mix alone);
- Negative control of the transcription (remaining RT-Mix + BCR-ABL PCR mix)
- Thermo-cycler program
- 1) 10 sec 100°C
- 2) 1 min 96°C
- 3) 3 min 58°C
- 4) 2 min 72°C
- 5) 10 sec 100°C
- 6) 20 sec 97°C
- 7) 25 sec 56°C
- 8) 25 sec 58°C
- 9) 10 sec 78°C
- 10) 90 sec 73°C
- 11) 31x from Step 5)
- 12) 10 min 73°C
- 13) forever 4 °C
- Agarose Gel Electrophoresis
- Prepare 2% agarose gel/GelRed, put into the chamber, and filled with buffer.
- Mix 7µl PCR product with 2µl loading buffer and add 7µl into the wells,
- Run the gel for about 25 (better 35) min at 80 volts (voltage for small gel).
- Take the image of the gel and print the picture.

5. *Leftover cDNAStorage*:

- The remaining cDNA templates should be stored at -20^{o}C using the according to storage worksheet.

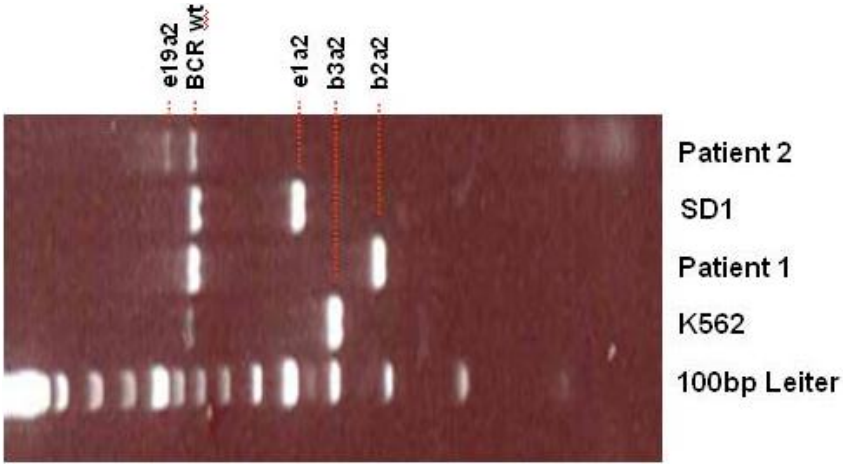
6. Result Interpretation: *type of transcript, primers & product size* (Cross NC et al., Leukemia 1994)

- ❖ M-BCR-ABL (b2/a2): (BCR-F4/ABL-R2) 310 bp
- ❖ M-BCR-ABL (b3/a2): (BCR-F4/ABL-R2) 385 bp
- ❖ m-BCR-ABL (e1/a2): (BCR-F3/ABL-R2) 481 bp
- ❖ µ-BCR-ABL (e19a2): (BCR-F4/ABL-R2) 927 bp
- ❖ WildtypBCR transcript alone! (no fusion BCR-ABL)normal cell: (BCR-F4/BCR-R1) 808 bp
- ❖ Rare transcripts:
- ❖ (e6a2) (BCR-F3 / ABL-R2) 1125 bp
- ❖ (e8a2)(BCR-F3 / ABL-R2) 1319 bp

7. Quality control Tips!

➢ If the BCR band is missing, or IF the positive or negative controls failed, repeat Multiplex PCR &/or the cDNA synthesis with or without RNA extraction!

8. Schematic Explanation



Reagent and material used for q PCR

- Real-time quantitative PCR machine. (e.g., Applied Biosystems 7900HT Fast real-time PCR system).
- l 96-well semi skirted PCR plates.
- Optically clear adhesive film. l
- PCR Workstation. l
- TaqMan fast advanced master mix (Life Technologies).

- ERM-AD623 BCR-ABL pDNA CALIBRANT [24]. –
- The 6 plasmid solutions are available from the European Commission's Joint Research Centre (JRC).
- Primers and probes

HL-60 cDNA as Negative BCR-ABL1 Control [27]. l K562 cDNA as positive BCR-ABL1control. l High (10% BCR-ABL1) and low (0.1% BCR-ABL1) controls. The high and low QCs are prepared from dilution of K562 cDNA in HL-60 cDNA. New stocks of cDNA are tested to determine the copy number of ABL1 and BCR-ABL1. Cell line cDNA is then diluted to approximately 5×10^3 ABL1 copy number and retested against the standard curve. With HL-60 and K562 at the same copy number, 1 part K562 and 9 parts HL-60 can be combined to make the high QC. The high QC can then be used to create the low QC by combining one part high QC with 99 parts HL-60 cDNA.

The qPCR program

- 2 minutes for 50 C^O
- 10 minutes for 95 C^O
- 15 seconds for 95 C^O 50x
- 1 minute for 60 C^O

MasterMix: Taqman Universal PCR Mastermix No Amperase UNG-4324081, Applied Biosystem

Primer and probe

	Primer		Probe	
BCR-ABL:	Forward	Reverse		
e13a2/e14a2	ENF-501	ENR-561	ENP_541 FAM/TAMRA	
e1a2	ENF-402	ENF-561	ENP-541 FAM/TAMRA	
ABL:				
	T494 ENF_1003	T495 ENR_1063	ENPR 1043 FAM/TAMRA	
Reagent	Concentration	Volume1x	Final	Volume 10x

			concentrations	
H2O, PCR grade		5.50 µl		55.0 µl
Probe	10µM	0.50 µl	2 µM	5.0 µl
Primer forward	10 µM	0.75 µl	3 µM	7.5 µl
Primer reverse	10 µM	0.75 µl	3 µM	7.5 µl
PCRMaster Mix Universal	-	12.5µl	-	125.0µl
Volume pre tube		20.0 µl	-	200.0 µl
cDNA		5.0 µl		
Total volume		25 µl		

Annex II: Patient data Abstraction checklist from the patient clinical record

Part I: Presence of Co-morbid Illnesses

1. Are there any Co-morbid No □ Yes □conditions other than CML?

2. If yes for question number 1, what type of co-morbid condition/s is this/are they? _____

3. Is there any medications taken by the patient? Please list all the medications _____

II. What was initial blast count (Myeloblast percentage): _____

III. Stages of Disease at diagnosis

□ Chronic Phase □ Accelerated Phase □ Blast Crisis Phase

IV. Cytogenetic/BCR-ABL/ Ph chromosome test

□ Was BCR-ABL(RT-PCR) test done?

□ If yes what type of CML was it?_____

Ph chromosome Percentage:□ _____

□ If the test was not done, why? _____

V. Clinical Findings 1. Presence of splenomegaly: Yes□ No□

2. If yes, Spleen size below right costal margin: _____

3. Presence of hepatomegaly: Yes□ No□

VI. Prognosis based on

1. Sockal Prognostic Scoring system

Low risk (□<0.8) □ Intermediate risk (0.8-1.2) □ High risk (>1.2)

VII. Treatment History

4. Daily dose of Imatinib: 300mg□ 400mg□□ 600mg □ 800mg

Part III. Documented baseline and follow-up laboratory and clinical findings at and after treatment initiation.

Date: _____

1. Baseline findings

CBC	Normal Range	Lab finding	Remark
WBC count x 103 cells/ mm3			
Hgb, g/dl			
Clinical findings	Yes	No	
Splenomegaly			
Hepatomegaly			

2. Follow up findings

CBC	Normal Range	Lab finding	Remark
WBC count x 103 cells/ mm3			
Platelet count x 103 cells/ mm3			
Hgb, g/dl			
Clinical findings	Yes	No	
Splenomegaly			
Hepatomegaly			

3. About Regimen/dose change History

I. Was the dose/ regimen changed? Yes□ No□

II. If yes to Qn No I,

When (after initiation of Imatinib in weeks)_____ Why?

a. Dose decreased because the patient responds to the initial dose and changed to maintenance treatment.

b. Dose decreased because the patient didn't tolerate

c. Dosed increased, because the patient didn't respond to current treatment

d. Drug discontinued because patients have a deep molecular response to treatment

4. If the answer is “d”, Reasons for temporary treatment discontinuation _____

5. For how long, treatment discontinued (In weeks)_____

6. Supportive management given for the patient

1. _____
2. _____
3. _____

7. End of follow up Findings

a. Disease status:

□ Primary Relapse□

b. Disease Phase:

Chronic□ phase: Accelerated phase:□□ Blast Crisis phase:

c. Current Disease parameters

i. Current clinical status: Improved:□ The same:□ Worsened:□

ii. Was a Complete Hematologic response achieved? Yes□ No□□ Not Known

iii. Was molecular response achieved?

- no molecular response
- early molecular response
- major molecular response
- deep molecular response

Annex III: Information sheet for adults (≥18 years) and Consent form

Research Title: Evaluation of multiplex PCR as potential prognostic tools for CML patients in resource-limited settings at TASH, Addis Ababa, Ethiopia.

Student name: SaifuHailu

Introduction

My name is _____. I am conducting my study in, TikurAnbessaSpecialized Hospital, Hematology Clinic; Addis Ababa, Ethiopia and now I am an MSc student at the department of medical laboratory science. I am conducting my study on the evaluation of multiplex PCR as a potential prognostic tool for CML patients.

Objective

The main purpose of this study is to evaluate multiplex PCR as a potential prognostic tool for CML patients in resource-limited settings at TASH, Addis Ababa, Ethiopia. Your input will be extremely valuable as information will be used to assess your clinical finding with multiplex PCR results and to evaluate multiplex PCR as a potential prognostic tool for CML patients comparative with real-time quantitative PCR. This study will provide information for clinicians/hematologists on comparative prognostic tools for CML patients to initiate appropriate treatment and monitoring of the drug effect.

Risk

The study is carried out as part of the routine clinical services provided by the hematologists. The study protocol will be reviewed by the institutional review board at the college of health sciences, Addis Ababa University. All procedures with sample collection will be done as daily clinical sample management. About sample collection will be done first and foremost as part of the routine clinical management. No extra volume sample collection and unwanted procedures will be for the study. There is no considerable risk for participating in the study other than the possible minor bleeding from the site of puncture. The collection of specimens will be conducted by an experienced phlebotomist with strict sterile procedures to avoid the possibility of acquiring infection.

Procedure

The collection of peripheral blood samples will be conducted respectively to a standardized protocol, and processing of bone marrow samples will be performed according to a standardized protocol. 5 ml venous blood will be collected according to the regular clinical services; at the time of diagnosis and during follow-up time for those who start treatment. the sample will be stored for further test.

Expected Outcomes.

At the end of this study, we will evaluate multiplex RT- PCR as a potential prognostic tool for CML patients by detecting BCR-ABL translocation that will be done using the gel sizing method. Finally, our study paves the way for CML patient's multiplex reverse transcriptase PCR used as one of the prognostic tools for them. The study will identify types of translocations, molecular response, and treatment outcome and we will indicate the possible recommendations and may benefit you directly or indirectly by improving diagnostic outcomes for CML patients in the hospital.

Confidentiality

Each piece of information from you and your record would be completely confidential to the research and the data are stored with your name and only used for this study. None of this would affect the care you receive from the Hematology Ambulatory clinic of TASH but will help in future planning for the hospital. No identifying names or characteristics will go into my report, so you may share your thoughts openly. The clinicians will be responsible for the interpretation of the results and providing advice for further needs, in case of abnormal values.

Right to refuse or withdraw

We confirmed the best care provided for you if you are voluntary to take part. Taking part in this study is completely voluntary. It will be your choice whether to participate or not.

Contact person

If you have any questions, you may ask the person to whom you are giving your sample or the Principal investigator (PI), SaifuHailu

Addis Ababa University, CHS, School of public health, Department of medical laboratory science

Email: seifuhailuchala@gmail.com

Informed consent form:

English version

I, _____, agree to participate in the study evaluation of multiplex PCR as prognostic tools for chronic myeloid leukemia patient relative to real-time quantitative PCR. I have read and fully understand the statement for patients' information, plus I have the chance to ask any question related to the evaluation.

To take part in this study, I agree to drawn 5ml of whole blood from the venous at a time of diagnosis or follow-up. The collection of whole blood samples will be performed according to a standardized protocol. I consider the puncture may cause a little amount of discomfort. Cause a small amount of temporary discomfort. I understand that these samples will be used for this study purpose. All information related to my sample will remain confidential and will not be used against my privacy. I understand taking part in this study is completely voluntary. I understand that the study does not have any impact or risk to my medical condition.

Name of Participant_____ Date_____

Signature _____

Annex IV: Data collection from patient interview socio-demographic characteristics

Age_____ (in years)

Sex

Male □ Female □

Place of residence

□ Addis Ababa □Oromia □ Tigar □ SNNP □ Ahmar □ Somalia

□ Afar □Harari □Gambela □Benishangul □ Dire Dawa □ others

Specify the place _____

Occupation

Unemployed □ House □ wife Student□ Farmer□ Daily laborer□

Merchant□Employed □ Retired□ Other(s) [Specify]

_____

Annex V: የአማርኛ መጠይቅ ቅፅ

ቅጽ 1: የጥናቱ መረጃ ቅጽ

ቀን፦_____

ውድ የ ቃለ መጠይቅ ተሳታፊ፤እንደምን አደሩ/ዋሉ? የጥናቱ መግቢያ ስሜ _____**ይባላል። በ ጥቁር አንበሳ ስፔሻላይዝድ ሆስፒታል ሄማቶሎ ጅክፍል ዉስጥ አዲስ ወይም በተመላላሽነት** Imatinib (Gleevec) **የተባለዉን መድኃኒት ለጀመሩየ** CML**ህመ-ማን ስለየበሽታዉ በድነኤ ደረጃ ምን አይነተ ለዉጥ አምጥቶል የሚለዉን በመልቲፕሌስ** PCR **ማጥናት እና** Evaluation Of Multiplex PCR as Aprognostic Tools for Chronic Myeloid Leukemia Patients **ወደፊት መሆን እንደሚችል ማሳየት ይሆናል።እኔም አንዱ ጥናቱ አባልነኝ።የድነኤዉ** translocation **አይነተ በደንብይ ጠናል።**

የጥናቱ አላማ፡-

የዚህ ጥናት ዋና አላማው Evaluation Of Multiplex PCR as Aprognostic Tools for Chronic Myeloid Leukemia Patients in Resource-Limited Setting atTASH **የህክምናዉ ዉጤት በፒሲአር ደረጃ ምን እንደ ሚመስል ማሳየት ይሆናል።የመፍትሄ ሀሳቦች ንማቅረ ብነው።**

ከጥናቱ የሚጠበቁው ጤቶች/ጥቅሞች

ይሄ Multiplex PCR as Aprognostic Tools for Chronic Myeloid Leukemia Patients **ማገልገል እንደሚችል ማሳየት እና ታማሚዉ ላልተፈለገ ወጪ እንደይዳረጉ መቀነስ ነዉ።የመፍትሄ ሀሳቦችን ማቅረብ ነው። በተጨማሪም ከጥናቱ በሚገኙ ግኝቶች ይበልጥ ህክምና ዉጤትን በተወሰነ መልኩ ለማሻሻል እንደሚቻል መድረግ፤እርስዎ የጥቅሙ ተቋዳሽ ይሆናሉ ብለን እናምናለን። ስለዚህ የእርስዎ ቅንና ሓቀኛ መረጃ ለጥናቱ እጅግ በጣም ወሳ ኝነው።**

የተከበረ ጊዜዎ ስለሰጡን እጅግ በጣም እናመሰግንዎታለን

ቅጽ 2፡በቃለ መጠይቅ ለመሳተፍ የፈቃደኝነት ቃል መቀቢ ያቅጽ

በ CML **ታካሚዎች ዉጤት እንዲሁም ተያያዥ ጉዳዮች ላይ ያጠነጠነ እና ከመድሃኒቱ አወሳሰድ እንዲሁም ከህክምናዉ ጋር ተያይዘዉ ያሉትግሮችን በመቅረፍ የበሽታዉን የህክምና ዉጤት ለማሻሻል አላማ አድርጎ የተነሳ ጥናት ነው። መሰብሰቢ ያቅፁም ይህንኑ በአላማ አድርጎ የተዘጋጀ ነዉ። በመሆኑም ከእርስዎ፡ ከእርስዎ ካርድ ላይ እና ከሀኪምዎ መረጃ ለመውሰድ እንፈልጋለን። በዚህ ጥና ትለመሳተፍም ሆነ ላለመሳተፍ መወሰንዎ በሆስፒታሉ ዉስጥ ለሚያገኙት ማንኛዉም አገልግሎት ላይ ምንም አይነት ተጽዕኖ የማይኖረዉ ሲሆን ተሳትፎዉን ምማቻረጥ ወይም ጥያቄዎችን አለ መመለስ ይችላሉ። በዚህ ጥናት የእርስዎ መረጃ ሙሉ በሙሉ በምስጥር የተጠበቀ ና**

ለምርምሩ አላማ ብቻ የሚዉ ልነው። በተጨማሪም የእርስዎ ተሳታፊነት በፈቃደኝነት የተመሠረተ ነው። የጥናቱ አላምውን ተረድተው ና ጊዜዎን ሰውተው ፤ከ 10-15 **ደቂቃዎች ለሚፈጅ ቃለ-መጠይቅ እውተኛው መረጃ ለመስጠት ፍቃደኛ በመሆንዎ በቅድሚያ አመሰግናለሁ። አዎ ከሆነ፣ያስፈርሙ እና ይቀጥሉ፤ካልሆነ ወደ ሚቀጥለዉ ተጠያቂይ ሂዱ።**

_____ _____ **የቃለ መጠይቅ**

የቀረበለት ሰው ፊርማ የቃለ መጠይቅ አቅራ ቢፊርማ

በ የትኛው ጊዜ ጥያቄ ካለዎት ሰይፉ ሁይሉ ብለው በስ.ቁ. (+251)-984986053 **ወይምበኢ-ሜይል** eifuhailuchala@gmail.com **ይጠይቁን።**

የተከበረ ጊዜዎን ስለሰጡን እጅግ በጣም እናመሰግንዎታለን።

ዋና አጥኚ: አታላይሙሉ

አዲስአበባ ዩኒቨርሲቲ፣ ጤና ሳይንስ ኮሌጅ፤ ሜድካል ላቦራቶሪ ሳይንስ ትምህርት ክፍል

ኢ-ሜይል:seifuhailuchala@gmail.com

ስ/ቁ፡ (+251)-984986053

Socio-Demographic Characteristics and some clinical information from patients

1. **እድሜ**:_____**(በዓመት)**

   ፆታ:

   □ **ወንድ** □**ሴት**

2. **የመጡበትክልል**: _____**(በፅሁፍይፃፍ)**

3. **የሚኖሩበትቦታ**

   □ **አዲስ አበባ** □**ኦሮሚያ** □ **ትግራይ** □ **ደቡብ ሕዝብ** □ **አማራ** □ **ሶማሌ**

   □ **አፋር** □ ***ሀረሪ*** □ ***ጋምቤላ*** □ **ቤኒሻንጉል** □ **ድሬ ዳዋ** □ **ሌላ ከለ**

4. **ስራዎት ምንድ ነዉ**

   □**ስራአጥ**□**የቤትእመቤት**□**ተማሪ**□**አርሶ/አርብቶአደር**□**የቀንሰራተኛ**□**ተቀጣሪ**□**ነጋዴ**□**ጡረተኛ**□**ሌ ሎች** [**ይገለፀ**] _____

5. **የሚወስዱት መደሐኒት አይነት**

   □ hydroxurea____ mg □ imatinib ___mg □ nilotinib _____mg □ dasatinib__mg □ others__mg

6. ***መሀሁኒቱን ለስንት ግዝያት ወስደዋል***

□አዲስጀማሪ □ሶስትወረ □ስድስትወረ □አንድዓመት □ሌላከለየግልፁ

_____

7. መሀዳሀኒት አቋርጠዉ የዉቃሉ ለምን ያህል ግዜይግለጹ_____
8. አዎን ከሆነ መልሶ ለስድስተኛዉ መልስ
   ምክንያቶንይግለጹ
   በሃኪም ታዘዉ ወይ ስበሌላ ይጥቀሱ_____

Declaration

I, the undersigned, declare that this M.Sc. thesis is my original work, has not been presented for a degree in this or any other university, and that all sources of materials used for the thesis have been duly acknowledged.

M.Sc. candidate: SaifuHailu (B.Sc.)

Signature: _____ Date of submission: _____

This MSC thesis has been submitted with our approval as advisors.

Samuel Kinde MSc, the Ph.D. candidate. Biochemistry, Addis Ababa University, Addis Ababa, Ethiopia.

Signature ----------Date----------

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