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SYBR Green PCR is Cost-Effective for Detecting Genetic Abnormalities in Acute Myeloid Leukemia

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Abstract

Objective: To determine whether SYBR Green PCR is cost-effective for detecting genetic abnormalities in acute myeloid Leukemia or not.
Methodology: This cross-sectional study was conducted at Department of Pathology-CMH Lahore medical college and institute of dentistry from October 2023- March 2024. DNA samples from 70 adults diagnosed as AML at various hospitals were ran on RT-PCR using dye and probe-based methods for detection of genetic abnormalities in acute myeloid leukemia (AML). Data was analyzed by SPSS 23 and presented in frequencies and percentages.
Results: In a total of 70 patients, twenty-five (17.5%) had defining genetic abnormalities. Fusions were detected in 19 (13.3%) patients while three rearrangements (2.1%) and three mutations (2.1%) were detected. The probe-based RT-PCR matched results in all patients.
C**onclusion**: SYBR Green PCR is very cost-effective for detecting genetic abnormalities in acute myeloid leukemia.
Key words: SYBR Green based RT-PCR, Acute myeloid leukemia.

Introduction

Within few years diagnosis of acute myeloid leukemia (AML) will be based on detection of fusions, rearrangements, and mutations by using Next Generation Sequencing or Real time- Polymerase Chain methods.[1] TaqMan™ Probe based assays to detect purposed acute myeloid Leukemia with defining genetic abnormalities are sensitive, specific but very expensive.[2] In order to make this tool affordable while maintaining the standards requires experimenting with low-cost PCR methodologies. SYBR-Green fluorescence based Real-time PCR is an economical alternative to the TaqMan™ probe PCR. It can easily not only detect but also quantify gene rearrangements, gene amplifications and deletions.[3] So current study was designed to determine whether SYBR Green PCR is cost-effective for detecting genetic abnormalities in acute myeloid Leukemia.

Methodology

This cross-sectional study was conducted at Department of Pathology-CMH Lahore medical college and institute of dentistry after getting ethical approval from CMH Lahore medical college and institute of dentistry (Letter no. 690/ERC/CMH/LMC). Study duration was 6 months from October 2023- March 2024. Sample size was calculated by Raosoft software. Seventy patients who were diagnosed cases of Acute myeloid Leukemia with defining genetic abnormalities were included after taking informed consent and were retested with SYBR Green. Sample was run at Pathology laboratory of CMH Lahore Medical college.

PCR procedure: About 3 ml of venous blood was drawn into an EDTA tube. RNeasy plus mini kit (Qiagen, Germany) was used for extraction of pure Ribose Nucleic Acid (RNA). NanoDrop™2000c Spectrophotometer (Catalog No ND-2000,Thermo Scientific™). Ultraviolet absorption was used for checking exact concentration and quality of RNA samples in the range of absorption spectrum at 260/280 nm. Transcription of RNA (1 µg) was carried out by using Quantitect Rev kit for transcription (Qiagen, Germany). The complementary DNA (cDNA) was obtained (QuantiNova Reverse Transcription KitQiagen, Germany). SYBR Green real-time qualitative PCR was done in two step method by making serial twofold dilutions of cDNA to make a standard curve. About 25µl master mix with 2 µl of complementary (cDNA), primers forward and reverse 1.5 µl were added. Next step was the amplification of (Quantitect) SYBR Green master mix (Qiagen, Germany). The procedure devised comprised of 45 cycles of denaturation at 94 °C for 15 s each preceded by initial PCR activation at 95 °C for 15 min. Later primer annealing was done at 55°C for 30s followed by extension at 72°C for 30s. At the end of each cycle fluorescence was measured.

Quality control: We used a time-tested in-house control (IC)Glyceraldehyde-3-phosphate dehydrogenase (GAPH) in every run. GAPDH is occasionally utilized as an internal control both for qualitative and quantitative RT-PCR analysis. It has the unique benefit that its expression is the same at various points in time and in different experimental conditions.[4] Distilled water was used as negative control. As per standard guidelines each test was repeated in duplicate.

Validation of PCR by analysis of Melt curve: A melting curve analysis was performed after amplification for verification of specificity regarding products being amplified at their specified melting temperatures (Tm). Denaturation was part of the process and carried out at 95 °C for 15 s, later the temperature was decreased to 60 °C for 1 menthe temperature was raised to 95 °C slowly at a rate carefully adjusted to increase by 1% meanwhile recording of fluorescence was continuously done.

Figure 3 represents melting peak of KMT2A rearrangement at 87°C.Gathering of relevant data with result analysis was carried out with the help of Applied Biosystems (ABI) 7500 Prism System™ (Thermo Fisher Scientific USA) Software Version SDS 2.0.1. The threshold of fluorescence limit of the ABI Prism 7500 system was adjusted at 0.02 as recommended by the guidelines and advised by the manufacturer. Various PCR products obtained were monitored by direct detection using the measured making use of dye produced fluorescence by the DNA double strands produced after every PCR cycle. The Fusions included for detection were *PML: RARA, RUNX::RUNX1, CBFB:: MHY11, DEK:: NUP214, RBS15:: MRFTA and BCR:: ABL1.* Rearrangements included *KMT2A, MECOM* and *NUP98*. Mutations included *NPM1* and *CEBPA*.

Primer sequence: The sequence of Primers used were as follows, **PML-RARα**(F) 5'- AGAGGATGAAGTGCTACGCCT-3' (R) 5'-TTCCGGGTCACCTTGTTGAT-3'**RUNX1-RUNX1T1**(F) 5'-GTCGAAGTGGAAGAGGGAAA-3'(R) 5'-CACAGGTGAGTCTGGCATTG-3'**CBFB-MYH11**CBFB 5'-GCAGGCAAGGTATATTTGAAGGC-3'MYH11 5'-CTTCCAAGCTCTTGGCTTTCTTC-3'**DEK-NUP214** DEK 5′-AGCAGCACCACCAAGAAGAAT-3′NUP214 5′-GTCTCTCGCTCTGGCACAAG-3′**RBM15-MRFTA**(F) 5'-CTTCATGCCTTCCCACCTT-3' (R) 5'-TCCAAGCTCCTTCTCTGCTC-3'Eukaryotic18S rRNA: Hs99999901_s1 **ICBCR-ABL1**BCR-ABL1 BCR-1F 50-CGCATGTTCCGGGACAAAAGC-30ABL1-1R 50-CGAGCGGCTTCACTCAGACC-30BCR-2F 50-AACAGTCCTTCGACAGCAGCAGTC-30ABL1-2R 50-CAGACCCTGAGGCTCAAAGTCAGA-30**KMT2A**(F) 5'-GTGCTTTGTGGTCAGCGGAAGT-3'(R) 5'-TGTGAGACAGCAACCCACGGTG-3'**MECOM**(F) 5'-CCTTATGTGGGAGAGCAGAGGT-3'(R) 5'-GAAGGCTATTCCTACGTCTGAGC-3'**NUP98**(F) 5'-GGAACCTGTGTCTGCCTCAACA-3'(R) 5'-CTTTGGAAGGCAGGCGACTGAA-3'**NPM1**(F) 5'-GCCAGTGCATATTAGTGGACAGC(R) 5'-GGAACCTTGCTACCACCTCCAG**CEBPA-α**(F) 5'-AGGAGGATGAAGCCAAGCAGCT(R) 5'-AGTGCGCGATCTGGAACTGCAG.**Internal control (IC)Glyceraldehyde-3-Phosphate Dehydrogenase**(F)5′-CTGAGTATGTGGTGGAGTCC-3′(R)5′-GAGGCATTGCTGACAATCTTG-3′**Negative Control** Distilled water.

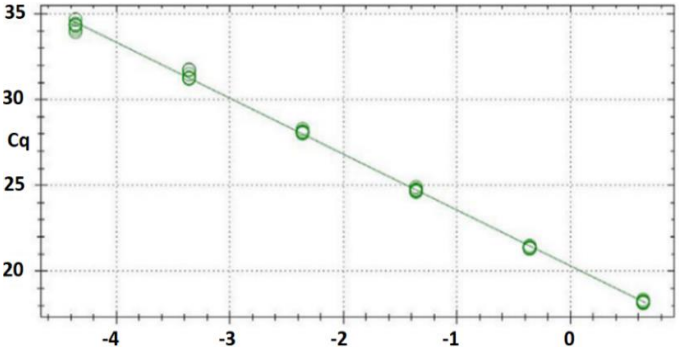


Figure 1. The assay linearity and limit of detection.

O – Standard, X – Unknown, _____SYBR E=102.8% R=0.999 Slope=3.25 y-int=20.318 (ABI 7500 System)

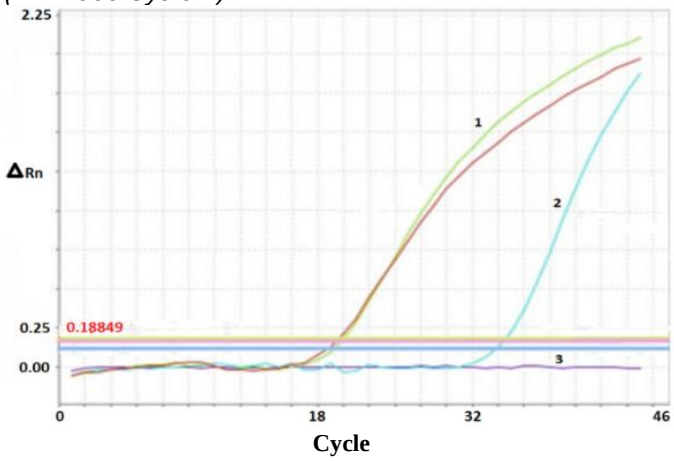


Figure 2. Amplification curves

(ABI 7500 System) Target genes=1, IPC= Internal Positive Control=2, NTC= Negative control=3.

Results

The mean age of patients was 68±15 years. Male to female ratio was 3:1. Twenty-five samples (17.5%) had defining genetic abnormalities with fusions being common (Table I). Fusions were detected in 19 (13.3%),

three rearrangements (2.1%) and three mutations (2.1%). The number of fusions which detected included PML::RAR1 (n=7, 10%), RUNX::RUNX1(n=2, 2.8%), CBFB::MYH11 (n=7, 10%) and BCR::ABL1 (n=3, 4.2%). No DEK::NUP214 and RBM15::MRFTA fusions were detected. As for rearrangements KMT2A (Figure 4) was detected in eight samples (n=3,10%). No MECOM and NUP98 rearrangements were detected. NPM1 mutation was detected in three samples (n=3, 10%) while no CEBPA mutation was detected. None of the patients had multiple defining genetic abnormalities.

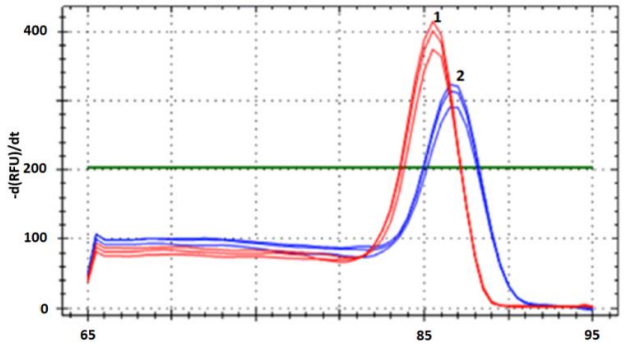
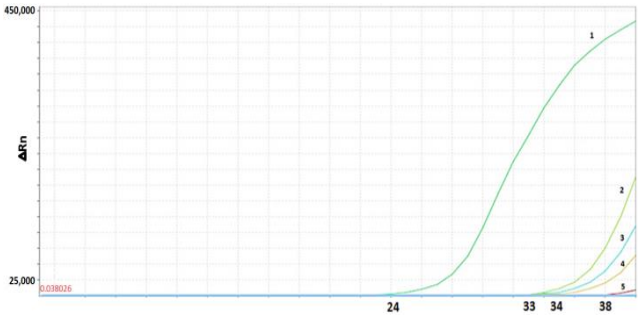


Figure 3. Melting curve.

Melt Peak
Temperature
Melt Peak at 87oC for KMT2A rearrangement(2)
(ABI 7500 System)

Table I: Genetic abnormalities in AML by SYBR green PCR (n=70)	
Genetic abnormality	No(%)
Patients showing defining genetic abnormalities	25(17.5)
Gene fusion abnormalities	19(33)
Gene rearrangements	3(2.1)
Gene mutations	3(2.1)



Ct (ABI 7500 System)

Figure 4. RT-PCR (ABI 7500) using SYBR Green I for detection of KMT2A rearrangement.

Discussion

In our landmark pilot study in Pakistan, we found out that "Cost per test of RT-PCR using dye SYBR Green for identification of genetic abnormalities of acute myeloid leukemia abnormalities was Rs 1093 (3.83$) versus almost at more than double the cost of Rs 2473 (8.67$) for TaqMan probe-based technique".[6]

We experienced complete conformity of results with expensive TaqMan probe-based PCR for these mutations run at two different centers. Difference of Rs 1380 (4.84$) per test is quite significant keeping in view that sensitivity and specifity of two methods is the same. This cost difference is because probes are expensive. Both SYBR Green I and TaqMan probes give equal number of tests and used quantity for both is similar. For developing and under resourced countries SYBR Green I based methods may be used instead of probe-based RT-PCR where feasible.[7]

There are so many benefits for using SYBR-Green-I for detection of molecular hematological aberrations TaqMan™ probe-based methodologies over probe-based methods. The biggest advantage is the cost benefits. The dye-based method is quite cheap and does not require the synthetic specific probes. Of course, like all methodologies SYBR-Green-I the major disadvantages of this technique is the "dimer" formation of any double stranded DNA which includes various non-specific polymerase chain reaction and various molecular products including primer-dimer formed which may lead to false positive reactions. It is significant if the final outcome is dependent exclusively data on regarding amplification. To avoid such discrepancies, it is most important to control the specificity signal of fluoresce signal by analysis of melting curve at the end of the PCR cycle.

In the coming era of precision medicine in diagnosis of acute myeloid leukemia molecular diagnostics will play a major role.[8] WHO established a precise classification of AML on the basis of molecular lesions (Table II).[9]

Until and unless we develop this capacity the underdeveloped world will be unable to reap the rewards of the precision medicine methodologies. Latest edition of World Health Organization describes molecular classification of Haematolymphoid Tumours (Fig 5) including fusions, rearrangements and mutations. We in our study did not cover AML myelodysplasia related and other defined genetic abnormalities however relevant fusions, rearrangements and mutations were covered in our study.[9-11]

Table II: Acute Myeloid Leukaemia with defining genetic abnormalities

Acute myeloid leukaemia with defining genetic abnormalities
Acute promyelocytic leukaemia with *PML::RARA* fusion
Acute myeloid leukaemia with *RUNX1::RUNX1T1* fusion
Acute myeloid leukaemia with *CBFB::MYH11* fusion
Acute myeloid leukaemia with *DEK::NUP214* fusion
Acute myeloid leukaemia with *RBM15::MRTFA* fusion
Acute myeloid leukaemia with *BCR::ABL1* fusion
Acute myeloid leukaemia with *KMT2A* rearrangement
Acute myeloid leukaemia with *MECOM* rearrangement
Acute myeloid leukaemia with *NUP98* rearrangement
Acute myeloid leukaemia with *NPM1* mutation
Acute myeloid leukaemia with *CEBPA* mutation
Acute myeloid leukaemia, myelodysplasia-related
Acute myeloid leukaemia with other defined genetic alterati

SYBR is preferred for its *Cost-Efficiency:*[12]SYBR Green is significantly less costly and convenient than specific fluorescent probes. This can be a significant factor, especially when running high-throughput experiments or on a tight budget. *Versatility:*[13] SYBR Green can be used to detect any double-stranded DNA, making it a versatile choice for a wide range of applications. In contrast, methods using probes require special probes designed for the target sequence.

Simplicity of Assay Design:[14] Designing a SYBR Green-based qPCR assay is often simpler because it doesn't require the design and synthesis of specific probes. This can save time and effort in the experimental setup. *Flexibility in Primer Design:*[15] Thus, both SYBR Green-based quantitative PCR (qPCR) and probe-based qPCR are widely used techniques for detecting and quantifying specific DNA sequences. Each method has its own advantages and considerations, and the choice between the two depends on various factors, including the specific application and experimental design.

Conclusion

SYBR Green PCR is sensitive and very cost-effective for detecting genetic abnormalities in acute myeloid leukemia.

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