CODEN [USA]: IAJPBB **ISSN: 2349-7750**

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

http://doi.org/10.5281/zenodo.2598031

Available online at: http://www.iajps.com ***Research Article***

Clinical utility of ARMS-PCR amplification-refractory mutation system for the detection of JAK2 gene variations [JAK2 haplotypes] in myeloproliferative disorders [polycythemia vera, essential thrombocythemia, and primary myelofibrosis in Tabuk.

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Article Received: December 2018 **Accepted:** February 2019 **Published:** March 2019

Abstract:

Background:

The germline JAK2 haplotype known as "GGCC or 46/1 haplotype" [haplotype$^{GGCC_46/1}$] consists of a combination of single nucleotide polymorphisms [SNPs] mapping in a region of about 250 kb, extending from the JAK2 intron 10 to the Insulin-like 4 [INLS4] gene. Four main SNPs [rs3780367, rs10974944, rs12343867, and rs1159782] generating a "GGCC" combination are more frequently indicated to represent the JAK2 haplotype. These SNPs are inherited together and are frequently associated with the onset of myeloproliferative neoplasms [MPN] positive for both JAK2 V617 and exon 12 mutations. Therefore the aim of our study was to optimize ARMS-PCR system for the detection of JAK2 haplotypes i.e rs3780367T/G and rs12343867 T>C in Myeloid proliferative disorders

Methodology:

This study was conducted on 88 samples among which 44 were MPD patients and 44 were healthy controls. DNA was extracted by Qiagen Kit and ARMS PCR system was optimized to detect JAK2 haplotype in Myeloid proliferative disorders .

Results:

The study included 88 specimens among which 44 were newly diagnosed MPD patients .Out of 44 MPD patients 26 [59%] were polycythemia vera, 17 [39%] Essential thrombocytopenia and 1 [2%] primary myelofibrosis and 44 healthy controls. Of 44 consecutive MPD patients, 14 [31.81%] were below or equal to 40 years age and 30 [68%] were above 40 years of age. Of 44 consecutive MPD patients, 30 [38.63%] were males and 27 [61.36%] were females. Among healthy controls 22 [50%] were below or equal to 40 years age and 22 [50%] were above 40 years of age. Of 44 healthy controls, 23 [51%] were males and 21 [49%] were females. ARMS PCR system was optimized to detect JAK2 haplotypes rs3780367 T/G and rs12343867 T>C gene variations in Myeloid proliferative disorders. The technique was successfully optimized by using wild-type or mutant-type primers with matched or one-base mismatched to examine the known SNPS in JAK2 gene. It was indicated that ARMS technique can be used as a potential molecular tool in the detection of potential JAK2 gene variations in MPDs .

Conclusion:

In our study, we successfully developed the Allele specific ARMS PCR technique for rapid detection of SNPs in JAK2 gene. Taken together, ARMS could be useful for quick and accurate SNPs detection in JAK2 gene in MPD as well as other genetic diseases in undeveloped and developing countries which are in shortage of medical resources and supplies.

Keywords:

Myeloproliferative disorders [MPD] ARMS-PCR amplification-refractory mutation system ,SNP- Single-nucleotide polymorphism, polycythemia vera [PV], essential thrombocythemia [ET], and primary myelofibrosis [PMF]

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Please cite this article in press Rashid Mir et al., ***Clinical Utility Of Arms-Pcr Amplification-Refractory Mutation System For The Detection Of Jak2 Gene Variations [Jak2 Haplotypes] In Myeloproliferative Disorders [Polycythemia Vera, Essential Thrombocythemia, And Primary Myelofibrosis In Tabuk..,*** *Indo Am. J. P. Sci, 2019; 06[03].*

INTRODUCTION:

Myeloproliferative neoplasms [MPNs] are a group of clonal haematological disorders where there is a primary disorder at the level of the multi-potent haematopoietic stem cell leading to increased production in one or more blood cell types, characterized by increased production of mature blood cells [1-2]. Philadelphia chromosome-negative consist of polycythemia vera [PV], essential thrombocythemia [ET], and primary myelofibrosis [PMF]. A number of stem cell derived mutations have been identified in the past 10 years. These findings showed that JAK2V617F, as a diagnostic marker involving JAK2 exon 14 with a high frequency, is the best molecular characterization of Ph-MPNs. Somatic mutations in an endoplasmic reticulum chaperone, named calreticulin [CALR], is the second most common mutation in patients with ET and PMF after JAK2 V617F mutation [3-4]. Discovery of CALR mutations led to the increased molecular diagnostic of ET and PMF up to 90% [5]. The discovery of *Janus kinase 2* [*JAK2]* mutation make it easy to diagnose polycythemia vera [PV] in which the prevalence of this mutation is reported up to 96%. Multiple research groups [2005] with an interest in myeloid neoplasia reported a new and exciting insight into the pathobiology of myeloproliferative disorders [MPDs]: the widespread occurrence of a somatic, acquired point mutation in a highly conserved residue of the autoinhibitory domain of the Janus kinase 2 [JAK2] tyrosine kinase [6]. Most series used the 2001 World Health Organization clinicopathological diagnostic criteria for hematopoietic neoplasms [7] as the "gold standard" for purposes of determining sensitivity and specificity of the JAK2 mutation. This mutation results in a valine to phenylalanine substitution at codon 617 within the Jak homology domain 2 [JH2] pseudokinase domain of JAK2. Expression of the JAK2V617F kinase in vitro demonstrates constitutive activation and factor-independent growth, [8] and expression of JAK2V617F in a murine bone marrow transplant model results in erythrocytosis in recipient mice , [9] These data suggest that JAK2V617F is a constitutively active tyrosine kinase and that activation of the JAK2 tyrosine kinase by the V617F mutation is an important pathogenetic event in PV, ET, and PMF [10]. Four main SNPs [rs3780367, rs10974944, rs12343867, and rs1159782] generating a "GGCC" combination are more frequently indicated to represent the *JAK2* haplotype. These SNPs are inherited together and are frequently associated with the onset of myeloproliferative neoplasms [MPN] positive for both *JAK2* V617 and exon 12 mutations. Association of *JAK2* alleles and/or haplotypes with MPNs has now been reported in Caucasians [11], Japanese [12], Chinese [13] and Brazilians [14]. However, work remains to be done to identify the causal variants in or flanking the *JAK2* locus and to delineate the mechanism by which such casual variants contribute to MPN development.

Although there are many sophisticated molecular biology techniques applied to clinical diagnosis, it is very expensive for people in undeveloped countries. As for known mutations in genes, ARMS PCR provides an optional way to solve this problem with its additional characteristics such as time-efficient, unbiased, sensitive, accurate, rapid, and reliable. Previously, β-thalassemia prenatally was diagnosed by successfully using ARMS-PCR system in China and in Iran [Fu et al and Moghadam et al], respectively; [15-16]. Chiu et al utilized ARMS-PCR method to quantify given mutant mtDNA heteroplasmies,[17] whereas Newton et al applied ARMS for antenatal diagnosis of cystic fibrosis.[18]Recently, Singh et al performed prenatal gene diagnosis for sickle cell disease by ARMS.[19] More recently, Aquino et al performed ARMS-PCR to detect the most common mutations of the *CFTR* gene in Peruvian patients with cystic fibrosis. [20]. ARMS and DNA sequencing was combined for the diagnosis of β-thalassemia in East-Western Indian population for better management [Shah et al [21]. From the above instances, it is noteworthy that ARMS can be used in the pre- and postnatal diagnosis of various genetic diseases. [22]. although there are many sophisticated tools applied to clinical diagnosis, it is very expensive for people in western China and some other undeveloped countries. As for known mutations in genes, ARMS provides an optional way to solve this problem with its additional characteristics such as time-

efficient, unbiased, sensitive, accurate, rapid, and reliable. Therefore the aim of our study was to evaluate the incidence of JAK2 gene variation rs3780367 T>G and JAK2 rs12343867 T>C in Myeloid proliferative disorders.

MATERIALS AND METHODS:

Study Population: This study was done on 88 specimens among which 44 were histologically confirmed MPD patients and 44 healthy controls .This research study was approved by the Research ethics committee of University of Tabuk and written informed consent was obtained from all the subjects before enrollment.

Inclusion criteria: The study included myeloproliferative disorders [Polycythemia vera [PV], essential thrombocytosis [ET], myelofibrosis [MF].All cases of MPD with Ph negative. All participants were of Saudi origin.

Exclusion Criteria: Patients with any other significant malignancy. Ph positive MPD patients and Nonsaudi Arabian cases were excluded.

Sample collection

After obtaining a written informed consent form and assessing the clinicopathological findings, a 3ml sample of peripheral blood was collected by venipuncture in EDTA tubes from each patient.

Sample Size: The study was conducted on 88 specimens among whom 44 were clinically confirmed MPD patients and 44 were healthy controls.

Genomic DNA extraction: The DNA extraction was done by using DNeasy Blood Kit cat 69506 from Qiagen as per the manufactures instructions. The QIAamp DNA Blood Mini Kit was designed for processing up to 200µl fresh or frozen human whole blood. The DNA extracted was dissolved in nuclease-free water, and stored at 4°C until use. The DNA quality and yield was assessed using Nanodrop [optical density] and 1% agarose gel electrophoresis

Optimization of AS-ARMS-PCR JAK2 rs3780367 T>G: Genotyping for JAK2 rs3780367 T>G was performed on the genomic DNA using a tetra-primer ARMS PCR approach. The ARMS primers were designed by using Primer3 software as depicted in Table 1.

Table No 1: ARMS-PCR for JAK2 rs3780367 T>G			
F1		5'-AGAAAGATAGCATGAAGAATTATCAAGG-3'	354bp
R1		5'-CGATGTAGTGCCAGTATAGTGATAGAAG-3'	
F2	T	5'-GCTTTATCTTATAAATGATGGAAAACTGTT-3'	178bp
R2	G	5'-ATAATTAATAGCAACAATACCATCCCTC-3'	234bp

ARMS primers were not working properly so we converted them to allele specific PCR as shown in table 2.

Table No 2: AS-PCR for JAK2 rs3780367 T>G			
AS-PCR for JAK2 rs3780367 GG genotype			
F1		5'-AGAAAGATAGCATGAAGAATTATCAAGG-3'	234bp
R2	G	5'-ATAATTAATAGCAACAATACCATCC**CTC**-3'	
AS-PCR for JAK2 rs3780367 TT genotype			
F2	T	5'-GCTTTATCTTATAAATGATGGAAAACT**GTT**-3'	178bp
R1		5'-CGATGTAGTGCCAGTATAGTGATAGAAG-3'	

The cocktail was prepared for 5 samples as depicted in table 3 .The AS-PCR was performed in a reaction volume of 25uL containing template DNA [50ng], FO -0.25uL , RO -0.25uL, FI-0.25uL, RI -0.25uL of 25pmol of each primers and 10uL from GoTaq® Green Master Mix [cat no M7122] [Promega, USA]. The final volume of 25 uL was adjusted by adding nuclease free ddH_2O .Finally 2ul of DNA was added from each patient.

Table No 3: **Preparation of PCR cocktail**		
Reagent	**1x**	**5 x**
PCR master mix	5ul	25ul
Forward outer	0.25 ul	1.25 ul
Reverse outer	0.25 ul	1.25 ul
Forward wild	0.25 ul	1.25 ul
Reverse mutant	0.25 ul	1.25 ul
Nuclease free water	17.0 ul	85ul
Total volume	23ul	115ul

Optimization of AS-ARMS-PCR JAK2 rs12343867T>C

Allelic-specific tetra-primer amplification was performed on the genomic DNA using a tetra-primer ARMS PCR approach. The JAK2 rs12343867 T>C genotyping was detected by using amplification-refractory mutation system PCR .The ARMS primers were designed by using Primer3 software as depicted in Table 4. ARMS primers were not working properly so we converted it into allele specific PCR as shown in table 5.

Table No 4: ARMS-PCR system JAK2 rs12343867 T>C				
Fo1		TTACAATGCCCAAACAATAGAGTATTAT	348 bp	55 ^{0}C
Ro1		TACAATATCTAAGAAGCACAATAAAGCA		
FI-2	T allele	TTAATGGAACTGACAGAAATGATGAT	173 bp	
RI-2	G allele	CCAAAAAATATATCTAGTATCATATCACCG	230 bp	

Table No 5: AS-PCR for JAK2 rs12343867 T>C				
AS-PCR for JAK2 rs12343867 CC genotype 5'->3'				
F1		5-TTACAATGCCCAAACAATAGAGTATTAT	230 bp	55^{0}C
R2		CCAAAAAATATATCTAGTATCATATCACCG		
AS-PCR for JAK2 rs12343867 TT genotype				
F2	T allele	TTAATGGAACTGACAGAAATGATGAT	173 bp	
R1		TACAATATCTAAGAAGCACAATAAAGCA		

The AS-PCR was performed in a reaction volume of 25uL containing template DNA [50ng], FO -0.25uL , RO -0.25uL, FI-0.25uL, RI -0.25uL of 25pmol of each primers and 10uL from GoTaq® Green Master Mix [cat no M7122] [Promega, USA]. The final volume of 25 uL was adjusted by adding nuclease free ddH_2O .Finally 2ul of DNA was added from each patient.

RESULTS:

Study Population

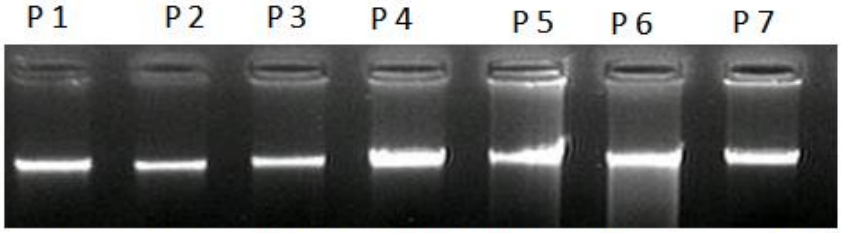
All demographic features of the subjects are depicted in table 6. This population-based case–control study was done on 44 subjects among whom 50 were clinically confirmed MPD patients and 50 were healthy controls with no history of any type of cancer.

Table.6: demographic features of MPD patients		
Number of cases	**N=**	**%**
Myeloproliferative neoplasms [MPNs]	44	
Polycythemia vera [PV]	26	59%
Essential thrombocythemia [ET]	17	39%
Primary myelofibrosis	1	2%
Age		
Age >40	30	68.18%
Age <40	14	31.81%
Gender		
Males	17	38.63%
Females	27	61.36%

The 44 newly diagnosed MPD patients among which 26 [59%] were polycythemia vera, 17 [39%] Essential thrombocytopenia and 1 [2%] primary myelofibrosis and 44 healthy controls. Of 44 consecutive MPD patients, 14 [31.81%] were below or equal to 40 years age and 30 [68%] were above 40 years of age. Of 44 consecutive MPD patients, 30 [38.63%] were males and 27 [61.36%] were females.

DNA extraction: The extracted DNA was dissolved in nuclease-free water and stored at 4° C until use. Quality and integrity of DNA were checked by NanoDrop™ [Thermo Scientific, USA]. The quality of DNA was also checked by running in 1% gel electrophoresis as shown in Figure 1.

Figure 1. :Genomic DNA preparation from peripheral blood of myeloproliferative patients

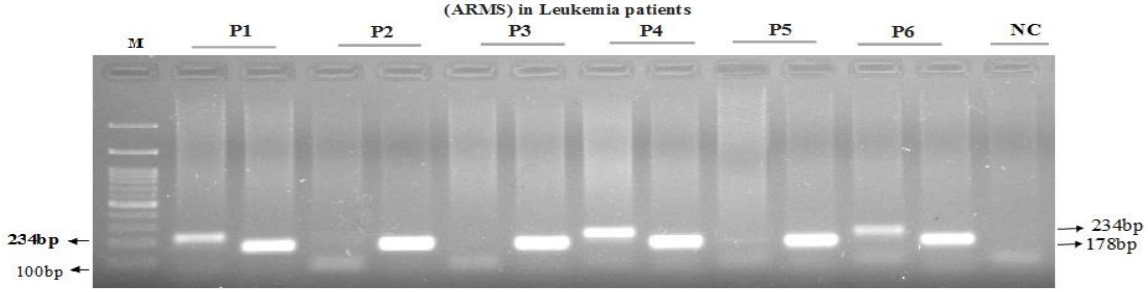


Genomic DNA preparation from peripheral blood of myeloproliferative patients

Optimization of JAK2 rs3780367 T>G gene variation

The JAK2 rs3780367 T>G genotyping was detected by using amplification-refractory mutation system PCR .But one tube reaction did not work properly then two tube reaction was performed .The amplification products were separated by electrophoresis through 2% agarose gel stained with 0.5µg/mL ethidium bromide and visualized on a UV transilluminator. Gradient PCR was performed .The best temperature was determined to be at 61°C in the temperature range of 55°C to 61°C tested with a gradient PCR thermocycler. The number of cycles was increased from 35 to 40 cycles, significantly enhancing the yields PCR products. Together, these changes resulted in a more robust amplification of the mutant allele and a less competing reaction from the control, as shown by the relative intensities of the corresponding bands on agarose gel electrophoresis. Primers F1 and R1 flank the intron 10 of JAK2 gene and amplify a wild-type allele [T allele], generating a band of 178 bp, and primers F2 and R2 generate a band of 234 bp from the mutant allele [G allele] as depicted in Figure 2.

Figure 2.: Genotyping of JAK2 rs3780367 T/G gene variation by amplification-refractory mutation system (ARMS) in Leukemia patients



Legend
M-100bp DNA ladder
Homozygous TT genotype –P2,P3 P5
Homozygous GG genotype-
Heterozygous T/G genotype –P1,P4,P6
NTC –Non template control

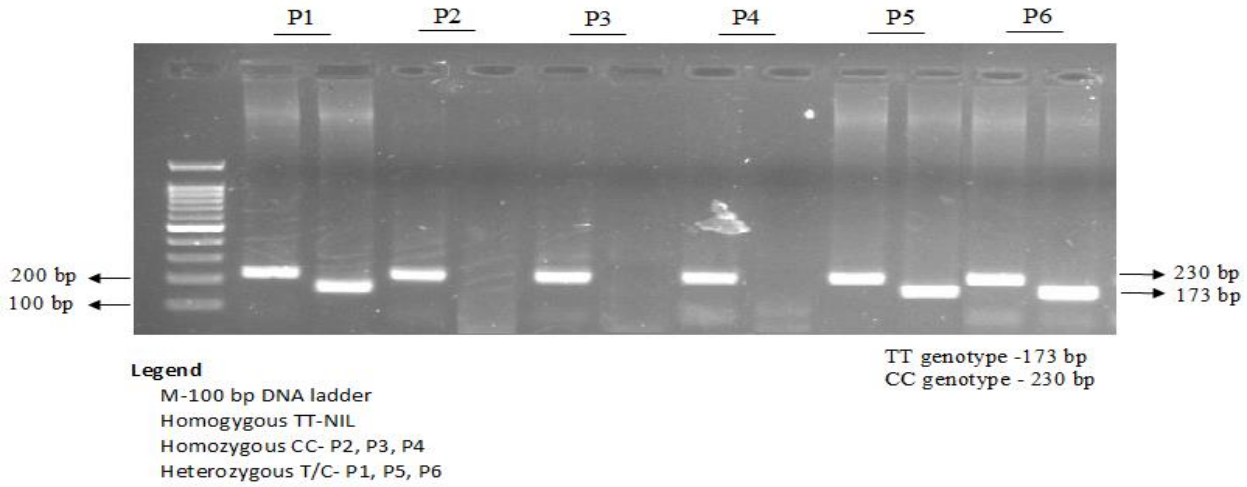
G allele - 234bp
T allele - 178bp

Optimization of JAK2 rs12343867 T>C gene variation

JAK2 rs12343867 T>C gene variation was studied by ARMS-PCR approach in myeloproliferative disorders [Polycythemia vera [PV], Essential thrombocythemia [ET], and Primary myelofibrosis [PMF]] .Allelic-specific tetra-primer amplification was performed on the genomic DNA using a tetra-primer ARMS PCR approach.But one tube reaction did not work properly then two tube reaction was performed .The amplification products of JAK2

rs12343867T>C genotyping were separated by electrophoresis through 2% agarose gel stained with 0.5µg/mL ethidium bromide and visualized on a UV transilluminator. Gradient PCR JAK2 rs12343867 T>C gene variation was performed and the best temperature was determined to be at 55°C in the temperature range of 52°C to 58°C tested with a gradient PCR thermocycler. Together, these changes resulted in a more robust amplification of the mutant allele and a less competing reaction from the control, as shown by the relative intensities of the corresponding bands on agarose gel electrophoresis. Primers F1 and R1 of JAK2 gene and amplify a wild-type allele [T allele], generating a band of 173 bp, and primers F2 and R2 generate a band of 230 bp from the mutant allele [C allele] as depicted in figure 3.

Figure 3: Optimization of JAK2 rs12343867 T>C gene variation in myeloproliferative disorders (Polycythemia vera (PV), Essential thrombocythemia (ET), and Primary myelofibrosis (PMF))



We successfully developed the Allele specific ARMS technique by using wild-type or mutant-type primers with matched or one-base mismatched to examine the known JAK2 gene variations.

DISCUSSION:

The *JAK2* V617F mutation is an acquired, somatic mutation present in the majority of patients with myeloproliferative neoplasms i.e. nearly 100% of patients with polycythemia vera and in about 50% of patients with essential thrombocytosis and primary myelofibrosis . The *JAK2* V617F mutation arises in a multipotent progenitor, is associated with erythropoietin-independent growth of erythroid progenitors, confers constitutive activity on the kinase, with enhanced downstream signaling, and was sufficient for the development of erythrocytosis in a murine retroviral model. Single nucleotide polymorphism [SNP], a novel molecular marker technology, refers to a sequence polymorphism caused by a single nucleotide mutation at a specific locus in the DNA sequence. This sort of polymorphism includes single base transitions, transversions, insertions and deletions, and the minor allele frequency should be 1% or greater. Of all the SNP mutation types, transitions are the most common [approx. 66.6%]. Currently, SNP markers are one of the preferred genotyping approaches, because they are abundant in the genome, genetically stable and amenable to high throughput automated analysis. Researchers in the past have used techniques like Polymerase Chain Reaction-Restriction Fragment Length Polymorphism [PCR-RFLP], direct DNA sequencing, Single Strand Conformation Polymorphism [SSCP],and tetra-primer Amplification Refractory Mutation System-Polymerase Chain Reaction [ARMS-PCR] for genotyping of specific polymorphic nucleotide loci [23-24]. Among these the tetra-primer ARMS-PCR could be a useful tool for genotyping, since SSCP may not be repeatable some times, direct DNA sequencing is a cumbersome, time consuming, technically demanding and costly procedure and the possibility of getting a restriction site for an enzyme could be rare for genotyping by RFLP.For genetic analysis, fast and economical assays that can be performed with standard PCR instruments are highly desirable. Compared to other genotyping techniques, such as typical PCR-RFLP analysis, real time PCR assay and DNA sequencing

AS-ARMS-PCR proved to be rapid, reliable, simple and economical assay for SNP genotyping. ARMS-PCR for JAK2 rs3780367 T>G , rs12343867 T>C gene variation can used routinely as it being rapid, reliable, simple and economical assay .

Previous studies concluded that although real time PCR and DNA sequencing are sensitive and accurate techniques, tetra-primer ARMS PCR assay could be beneficial in terms of total time, cost and applicability in a typical laboratory.

The assay described here for JAK2 rs3780367 T>G and JAK2 rs12343867 T>C is more convenient than the traditional PCR-RFLP since it eliminates the need for incubation with restriction enzymes. This not only avoids any consequent errors and artifacts from such procedures but also reduces the amount of DNA required for the digestion step in PCR-RFLP. No special equipment and only a small amount of standard PCR reagents are needed in tetra-primer ARMS-PCR [25-26]. Ye et al. [27] combined tetra-primer PCR with ARMS to form the tetra-primer ARMS-PCR or T-ARMS technique by introducing deliberate mismatches at position − 2 from the 3′ end of inner primers to improve allele specificity . ARMS-PCR or T-ARMS methods are rapid, simple, reliable, easy to perform, economical and require minimum level of expertise that can be used for both large- and small-scale genotyping studies. As for known mutations in genes, ARMS provides an optional way to solve this problem with its additional characteristics such as time-efficient, unbiased, sensitive, accurate, rapid, and reliable. In our study, we successfully developed the ARMS technique by using wild-type or mutant-type primers with matched or one-base mismatched to examine the known mutation in the JAK2 gene.

CONCLUSION:

In our study, we successfully developed the Allele specific ARMS PCR technique for rapid detection of SNPs in JAK2 gene in MPD as well as in other diseases in undeveloped and developing countries which are in shortage of medical resources and supplies.

Competing interests:-

The authors declare that they have no competing interests.

Authors' contributions:-

All authors read and approved the final manuscript. Thanks to all authors for their support and help in this study.

Acknowledgments:-

We would like to acknowledge the assistance of Mr. Mohammad Imran Uddin.

This work was supported by grants from the Deanship of Scientific Research [1430-0234] ,University of Tabuk. Saudi Arabia.

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