Diagnostic Accuracy of Different Methods in Detecting Factor V Leiden Mutation in Tertiary Care Centre

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ABSTRACT

Objective: To determine the diagnostic accuracy of clotting based APCR assay in determining Factor V Leiden mutation with PCR as gold standard and to establish the frequency of factor V Leiden mutation in patients presenting with thrombophilia as well as to study their clinical presentations and association with arterial or venous thrombosis.

Study Design: Cross sectional study.

Place and Duration of Study: Army Forces Institute of Pathology (AFIP), Rawalpindi Pakistan, from Dec 2018 to Sep 2019.

Methodology: A total of 230 patients were recruited which presented with DVT and other thrombotic conditions. Acquired causes of Factor V Leiden mutation were excluded from our study. For Screening tests APCR, blood was collected in 3.2% sodium citrate tubes while for PCR blood was collected in K2 EDTA tubes.

Results: On Screening, APCR test 44 (19.29%) showed positive results with cut-off value of <0.8 while 184 (80.70%) were negative. Out of total 230 patients, Factor-v mutation was detected in 42 (18.4%) of patients. On statistical analysis, Screening APCR was found to be 95.45% (95% sensitive CI: 83.84%-99.42%) and 97.85% (95%CI: 94.59%-99.41%) specific as compared to our gold standard method PCR. The positive likelihood ratio was 44.39 (95%CI: 16.76-117.03) while the negative likelihood ratio was 0.05 (95%CI: 0.01-0.19) with a positive predictive value and a negative predictive value of 91.30% (95% CI: 79.10%-96.35%) and 98.91% (95%CI: 95.92%-99.72%), respectively. The accuracy was 97.39% (95% CI: 94.36%-99.03%).

Conclusion: APCR for Factor-v Leiden Screening is a rapid and cost-effective method for diagnosis of Factor-v Leiden mutation.

Keywords: Factor-v, Leiden mutation

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INTRODUCTION

Venous Thromboembolism includes Deep vein Thrombosis and Pulmonary Embolism.[1] There is disruption of local blood flow as a result of clots formed in the deep veins of legs and these patients have tendency of having venous insuffiency and later on developing pulmonary emboli.[2] Venous Thromboembolism results from a complex interaction of environmental, genetic and acquired factors. The genetic risk factors include quantitative and qualitative abnormalities of naturally occurring anticoagulants including Protein C, Protein S or Antithrombin or defects causing an increase in the prothrombotic factors like Factor V Leiden mutation, Prothrombin mutation prolonged hospitalization or immobilization.[1]

Persons having a genetic predisposition have increased risk of developing thrombosis than normal population. Prevalance for FV Leiden mutation varies from 4-5% in the general population.[4] FV Leiden mutation may present with both arterial and venous thrombosis.[3,4]

Activated Protein C degrades activated factor V and VIII, thus acting as an anticoagulant and preventing thrombin production. Factor V Leiden results from a single point mutation in the Factor V gene.[5] This Guanine to Adenine transition at nucleotide 1691 in exon 10 of Factor V gene leading to substitution of glutamine for arginine at position 506. Other mutations in factor V have also been identified, however, Factor V Leiden mutation FVR506Q remains the most common. This mutation renders Factor V resistant to degradation by Activated Protein C. APC resistance is an important cause of heritable thrombophilia.[6]

In the laboratory, Factor V Leiden mutation is screened by an APTT based clotting test. Definitive diagnosis is by identification of the specific mutation by molecular methods. PCR based techniques not only identify the specific mutation but also establish the zygosity.[7,8]

In developing countries like Pakistan, health facilities are inadequate and the general population has limited access to good diagnostic facilities. Usually patients presenting with thrombosis are never screened

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for heritable thrombophilia which is an important cause. In countries with lack of finances and limited resources factor V Leiden APTT based clotting test is a useful screening tool. Facilities for PCR are only available at few referral centres, so use of Factor V Leiden screening test is cost effective method for the diagnosis of Factor V Leiden mutation. We have conducted this study with an aim to determine the diagnostic accuracy of clotting based APCR assay in determining Factor V Leiden mutation with PCR as gold standard. This will help to guide clinicians regarding the utility of this test. We also established the frequency of Factor V Leiden mutation in patients of our population presenting with thrombophilia. We have studied the association of this mutation with the clinical presentation and type and site of thrombosis.

METHODOLOGY

This cross-sectional study was conducted at Armed Forces Institute of Pathology (AFIP), Rawalpindi Pakistan, from December 2018 to September 2019 approved by IRB Committee with reference number (FC-HEM16-25/READ-IRB/17/380). Two hundred and thirty sample size was calculated according to WHO calculator with Z Score of 1.96%, 95% confidence level, Prevalance of 0.9% and Margin of error 0.09%.[9]

Inclusion Criteria: Patients diagnosed to have had thrombosis (established on clinical and radiological studies) were included in the study.

Exclusion Criteria: Patients having any known acquired major risk factor were excluded from the study. Patients currently on anticoagulant medications (warfarin and/or heparin) and having lupus anti-coagulants activity were also excluded.

Patients were informed about the study and written informed consent was taken. About 3 ml of blood was collected in EDTA and Trisodium citrate tubes. Platelet Poor Plasma was made by centrifuging at 1500 rpm for 10 mins. Screening of FVL was done along with Protein C and Protein S using PROC Global Test using kit Siemens Healthcare Diagnostics. In this procedure there is incubation of plasma with the protein C activator and contact phase activator which causes activation of Endogenous Protein C along with endogenous protein S. This activated Protein C and S causes inactivation of VIII and FV. The time taken for a clot to form WAS determined. This procedure was carried out according to instructions of Ca 1500 APCR kit. Values of <0.8 signify deficiency of protein C and S. If the values are more than >0.8 it indicates normal levels of Protein C and Protein S but if values are <0.8 there is deficiency of Protein C or Protein S. It needs than Protein S assay detection through factor V deficient plasma and protein C assay activity. For FVL screening test patient plasma was taken and mixed with factor V deficient plasma in the ratio of 1:4 andagain the test was performed with same reagent kit as described for ProC global. For PCR based analysis of FV Leiden mutation detected blood was collected in EDTA tube in concentration of 2-3ml of blood. DNA was extracted using Sol Gent DNA Prep kit. Extracted DNA was stored at -200 C. Amplication was done on conventional PCR Proflex using master mix 20ul, primer 1 ul, taq polymerase 0.1ul using 25 cycles. Amplification temperatures given in Table-I.

Table-I: Amplification Temperatures with reference to time.

Stage	Temperature	Time (secs)
Initial Denaturation	94	00 secs
Denaturation	94	00:30
Annealing	65	01:00
Extension	72	01:30
Final Extension	72	03:00min

Amplified product was then run on gel electrophoresis using silver nitrate staining. Each test was then compared with positive and negative control using SPSS version 24.

RESULTS

A total of 228 patients of thrombophilia having no acquired cause of thrombosis were studied over a period of 10 months. Median age was 23 with range of 1 to 35yrs. Male to female ratio is 2.3:1. Males were 160 (70%) and females were 68 (30%). Factor V Leiden was detected in 18.4% of cases. About 226 of total patients presented with venous thrombosis and 2 patients with arterial thrombosis. In our study most common clinical presentation includes DVT 98 (42.98%) followed by patients who presented with stroke 79 (34.64%), TIA 19 (8.33%), Cerebral venous thrombosis 11 (4.82%), Retinal venous occlusion 10 (4.38%), Pulmonary thromboembolism 3 (3.07%), Superior Mesenteric venous thrombosis 7 (3.07%) and Superior Sagital sinus thrombosis 1 (0.43%) as shown in Figure-1.

In our study out of 230 patients, 42 (18.42%) patients were positive for Factor V Leiden mutation and 186 (81.57%) patients were negative on PCR. In all these positive patients all other thrombophilic tests including Protein C, Protein S, Antithrombin, homocysteine and Prothrombin gene mutation were done and showed negative results. Out of 42 PCR positive patients 2 were negative on screening analysis and

labeled as False negative and finally picked by PCR mutation analysis Regarding Factor V mutation negative cases 186 patients showed negative results (Figure-2).

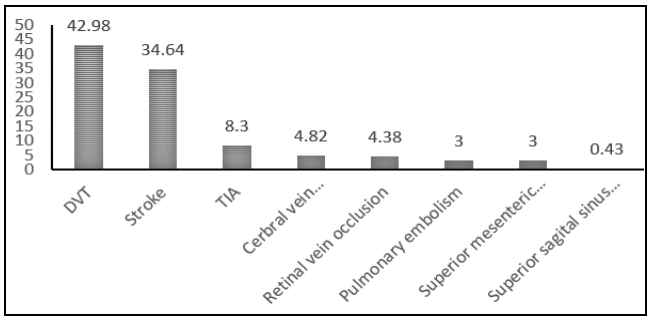

Figure-1: Thrombotic Episodes in patients.

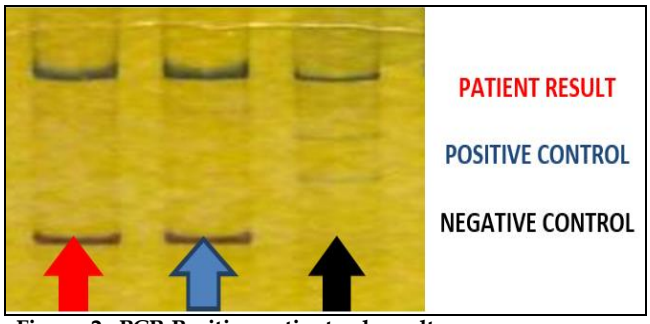

Figure-2: PCR Positive patient gel result.

Out of these negative results 182 were True negative while 4 were positive on screening analysis which but negative on PCR (Table-II).

Table-II: Comparison of Screening and PCR Gold Standard Results.

	Screening APCR	PCR (Gold Standard)
True Positive	40	42
True Negative	182	186
False Positive	04	00
False Negative	02	00

PCR for Factor V Leiden was done by gel detection method in two phases. In the first Round, sample was run to detect the presence of mutation. Column 1 and 2 show patient's sample run in duplicate along with positive and negative controls shown in column 3 and 4 respectively. It can be appreciated that patient sample matches the positive control for the mutation confirming the presence of Factor V Leiden mutation.

On Analysis using PCR Mutation Analysis Factor V Leiden mutation was detected in 42 (18%) of total patients and 186 (81.57%) were showed no mutation. In these positive patients 41 were heterozygous while only 01 homozygous mutation was detected. However, on Screening APCR test, 44 (19.29%) showed positive results with cutt-off value of <0.8 while 184 (80.70). On statistical analysis, Screening APCR was found to be 95.45% (95% sensitive CI: 83.84%-99.42%) and 97.85% (95%CI: 94.59%-99.41%) specific as compared to our gold standard method PCR. The positive likelihood ratio was 44.39 (95%CI:16.76-117.03) while the negative likelihood ratio was 0.05 (95%CI:0.01-0.19) with a positive predictive value and a negative predictive value of 91.30% (95%CI:79.10%-96.35%) and 98.91% (95%CI: 95.92%-99.72%), respectively. The accuracy of screening test was determined to be 97.39 % (95%CI:94.36%-99.03%) (Table-III).

Table–III: A 2x2 table showing comparison between two methods.

		APCR	APCR	Total
PCR	+	42	04	46
PCR	-	02	182	184
Total		44	186	230
p-value				<0.001

There is no statistical difference in the results of both methods

DISCUSSION

Considering the important role of Factor V Leiden mutation in thrombogenesis including hypercoagulability and increased tendency to develop venous thromboembolism, this study was done to compare the diagnostic accuracy of FVL mutation in heritable thrombophilia patients and also to discuss the frequency of Factor V mutation in our thrombophilia.[9,10]

Factor V has an integral and important role in coagulant as well as anticoagulant pathway. There is hypercoagulibity in Factor V Leiden due to increased coagulation and decreased anticoagulation. As a result of factor V Leiden mutation, Factor V becomes resistant from being cleaved and inactivated by activated protein C, a condition known as APC resistance resulting in more and more of Factor Va within the prothrominase complex, and thus increased coagulation and thus increased production of thrombin.[11,12] People who harbor the FVR 506Q mutation lack their cleavage product resulting in loss of anticoagulant activity from activated protein C. Protein S along with Activated Protein C helps increased coagulation and decreased anticoagulation thus contribute equally to the hypercoagulable state in factor V Leiden-associated APC resistance.[13,14]

Factor V leiden is the among the common cause of heritable thrombophilia. Its distribution is not regular worldwide. People of Africa, America and indigenous Australians donot have this mutation but White

Americans, Canadians and people of North European have 5% of this mutation and 15% in areas such as Sweden and Cyprus. Prevalence have been found to 2.5% in Asians and 1.9% in Saudi Arabs respectively.[1]

Laboratory tests can be screening and diagnostic assays. The screening tests are used to screen for the presence or absence of disease, while the diagnostic tests provide confirmatory evidence of disease.[15,16] Keeping in mind patients clinical history, Initial APCR screening followed by molecular workup of borderline positive cases, should be the sequence for the exact diagnosis. This will not only cut down costs by reducing unnecessary investigations but it fulfills the diagnostic criteria for diagnostic accuracy.[2] The APCR is a screening test, based on functional assay and number of confounding factors may affect the results.[13] The borderline positive cases should also be evaluated for elevated factor VIII activity levels, protein C or protein S abnormalities.[2]

A study conducted by Shaheen *et al,* according to which Heterozygous Factor V Leiden mutation was detected in 90 to 95% of cases with APC resistance and Homozygous mutation was detected in only small population as compared to heterozygous mutation and in these patients risk of venous thromboembolism is much greater.[11] This study results are much comparable with our study that showed 97% heterozygous while 2% homozygous results.

In a study conducted in Iran by Ghorbani *et al,* in 2018 that showed 100% compatible results for APCR screening result and PCR.[1] This same study was conducted after exclusion of history of anticoagulant drugs which showed that the very same patients who had Positive APCR results also had positive PCR results with 100% compatibility. In their study there are 26.7% cases which are positive for heterozygous mutation and 8.9% cases are homozygous,[1] that is much greater than our study in which there are 41 (97.61%) heterozygous cases and 1 (2.3%) homozygous cases.

In a study conducted by Rosendaal *et al,* in 1996 which showed Factor V Leiden hterozygous mutation in 18% of cases and homozygous mutation was detected in 1.5% of cases,[10] that is much lower than our results. In a study conducted by Zehnder *et al*, Factor V Leiden mutation was detected in 20% of cases.[12]

According to study conducted by Aboud *et al,* they showed APC 100% sensitivity and specificity for Factor V Leiden mutation and in our study sensitivity is 95.27% and specificity is 97.37%.[17]

PCR for F V Leiden testing is more sensitive and specific, which can replace functional testing for APC resistance however, PCR is more costly, more labor intensive and require more technical personel therefore, for the timebeing we cannot replace APCR with PCR, but PCR can be used to determine zygosity of mutations, for conformation of diagnosis in border-line cases and in patients who cannot stop anticoagulation therapy.[14,18]

PCR detects homozygous and heterozygous mutations specifically. It is not recommended to do only clot based screening test by the presence of factor V deficient plasma and directly do the PCR based genetic determination of Factor V Leiden Mutation as the genetic tests are more time consuming and more expensive as compared to clotting based screening test.[15]

The commonly used method for detection of factor V Leiden mutation involves a large step process including PCR amplification etc. These steps though are accurate and more precise but are labor intensive and because of their high cost values and no automation many international setups prefer to perform FVL genotyping by the new development of semi automated only one step homogenous method for the detection of Factor V Leiden mutation. These methods include e fluorescent detection of real-time PCR products with allele-specific hybridization probes, non-PCR signal amplification methods based on either enzymatic hybridization mismatch recognition using fluorescent allele-specific probesor linked fluorescent allele-specific pyrophosphorolysis-kinase reaction, and various other PCR and non-PCR based methods.[16] Patients of age >60 years workup of Protein C, Protein S, Factor V Leidene, Prothrombin gene mutation is unnecessary as most of these conditions present with venous thrombosis at early age.[18]

CONCLUSION

Based on our findings, we suggest that clotting based APC resistance assay is an effective screening test for factor V Leiden mutation. In resource-constrained countries, the PCR is an efficient tool to rule out thrombophilia due to factor V Leiden mutation. APCR was found be highly senstive and specific for the screening of FV Leiden mutation. APCR testing reaches to the sensitivity and specificity of 100% in homozygous Factor V Leiden mutation. In resource const-raint countries APCR can serve as an optimal screening strategy of Factor V Leiden while working for heritable thrombophilia cases.

Conflict of Interest: None.

Authors Contribution

SB: Direct Contribution to conception, design, analysis, interpretation, HMR: Intellectual contribution to analysis and data interpretation, AM; Analysis, data preparation, manuscript preparation, RM: Manuscript preparation, AK: Literature review and data collection, SAK: Literature review.

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