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HAPLOTYPYING FOUR THROMBOPHILIA MARKERS USING MultiGEN PLATFORM TECHNOLOGY

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SUMMARY

Using MultiGENtechnology we have developed a comprehensive assay to simultaneously detect three human genetic predisposition markers (Factor V Leiden, Prothrombin, and mthfr x2) from buccal swabs and blood. The MultiGEN assay is based on conventional Sanger sequencing technology, hence confirming the loci of the respective mutations. This one-time comprehensive test can better assist physicians in the management of women on oral contraceptives, hormone replacement therapy, and patients with other risk factors for cardiovascular diseases. patients with risk other factors for cardio vascular diseases

INTRODUCTION

Thrombophilia is the increased tendency to develop abnormal blood clotting which often manifests itself as a deep vein thrombosis (DVT) or pulmonary embolism (PE) . Hence, clot formation is a risk factor in the management of patients with a wide range of clinical conditions (e.g. cardiovascular diseases, pregnancy loss) and would be of interest to specialists in cardiology, internal medicine,oby/gyn, hematology, infertility etc.

Mutations in three genes: Factor V Leiden (1691), prothrombin (G20210A), and mthfr (C677T, A1298C) have been identified as playing key roles in the cascade of blood clotting. The magnitude of the abnormal clotting risk depends on the combination of these mutations, and the respective clinical conditions. For example, women who are on contraceptives and/or hormonal treatment can have a 35 times increased risk of developing DVT or PE (1,2, 3,4).

This manuscript presents the use of MultiGEN DNA sequencing technology in overcoming present technical limitations. Samples are taken with buccal swabs.Further, MultiGEN simultaneously detects all three states (mutant, wild and heterozygous) for Factor V Leiden, Prothrombin and two mthfr markers,and hence the relative cost per reported result is significantly less than other molecular diagnostic methods.

MATERIALS AND METHODS

Anonymous patient samples that had previously tested positive for Factor V, Factor II, mthfr –C677T and A1298C were obtained from reference laboratories. Total DNA was extracted using BugsNBeads A (NorDiag, USA). Multiple PCR of target amplicons was performed in a 50-ul volume containing 5ul of extracted DNA. The amplified multiple targets were purified using Ampure (Agencourt, USA). Purified amplicons were sequenced using MultiGEN sequencing primers by cycle sequencing using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit version 1.1 (Applied Biosystems, USA) on a GeneAmp 2400 thermocycler (PE Applied Biosystems, USA). Unincorporated dye terminators were removed using Clean Seq (Agencourt USA). The samples were then dried in a speed vac (DNA 120, ThermoSavant, USA) and re-suspended in 20 ul of Hi-Di formamide. Samples were analyzed by capillary electrophoresis using the ABI PRISM Genetic Analyzer 3130.

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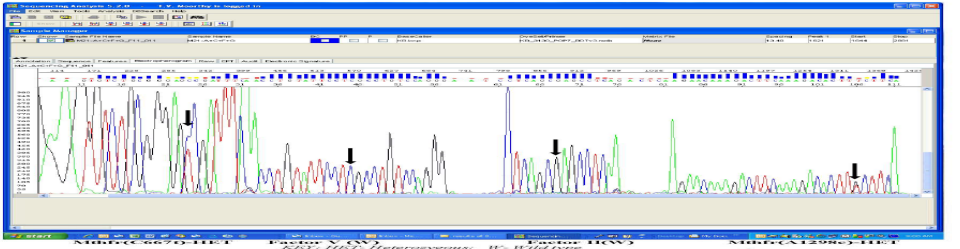

Fig.I SINGLE Electropherogram showing ALL FOUR mutations

THROMBOPHILIA MUTATIONS	
MARKER	MUTATION LOCUS
Mthfr (A1298C)	A G A A C G A A G A C T T C A A A G A C A C T T T/g C T T C A C T
Mthfr (C677T)	G C G G G A G C/t C G A T T T C A
Factor II(G20210A)	C T C A G C G/a A G C C T C A A
Factor V Leiden	C C T G T A T T C C T C/t G C C T G T T A G G G

Table I Locus of each Mutation

MARKER	ALLELE	MultiGen	PRESENT METHOD	INCIDENCE
Factor V	Wild G/G	12	12	63.15%
	HeteroG/A	5	5	26.30%
	Mutant A/A	2	2	10.50%
Prothrombin	Wild G/G	17	17	70.80%
	HeteroG/A	4	4	16.60%
	Mutant A/A	3	3	12.50%
Mthfr (C677t)	Wild C/C	10	10	40%
	Hetero C/T	14	14	56%
	Mutant A/A	1	1	4%

Table 2 Comparison of MultiGEN assay to present methods

RESULTS AND DISCUSSION

Four separate mutations in three key genes can affect blood coagulation. **The MutliGEN Thrombophilia Panel** simultaneously detects whether there are just one or two copies of each of these mutated genes. The normal state without mutations is also confidently identified. A total of sixty eight samples were analyzed, and their respective allele profiles are shown in Table 2. There were 19 samples for Factor V, 24 for prothrombin and 25 for mthfr. Most of them were wild type. Incidence of a single mutation was 29.4%, 16.6% and 56% respectively, and double mutations were 11.7%, 12.5% and 4% respectively. These results were in 100% concordance with results obtained with present methods.

Approximately one third of otherwise totally normal women have one or more mutations of genes that involve blood coagulation (Factor V Leiden, Factor 2 Prothrombin, and 2 mutations involving Mthfr). The **MultiGEN Thrombophilia Panel** identifies approximately 15% of the causes of fetal loss and infertility due to genetically determined coagulation abnormalities. Hence for conservative risk management in pregnancy, all women who are pregnant or contemplating pregnancy, experiencing infertility or unexplained fetal loss, and relatives and children of women who test positive are potential candidates for testing for these mutations. If there mutations are identified, various forms of anti-coagulation and other therapies (folic acid etc) are available depending on the mutation(s) detected, as well as prophylactic measures.

The principle value of genetic testing is to adopt measures that prevent or delay the onset of the respective clinical conditions. Hence genetic screening tests are more useful and appropriate for patients who may develop such conditions, rather than in patients who have already developed symptoms. Genetic predisposition testing for symptomatic patients is more for confirmation and identification of families at risk. MultiGEN has eliminated the inhibition of cost, and is therefore **well suited for large scale screening of pregnant mothers and women who are on contraceptive pills for the risk of abnormal blood clotting.**