

COMPARISON OF MUTAGEL HFE AND POLYMERASE CHAIN REACTION-RESTRICTION FRAGMENT LENGTH POLYMORPHISM (PCR-RFLP) FOR THE INVESTIGATION OF C282Y AND H63D POINT MUTATION IN HFE GENE

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Date submitted: March 29, 2013

Date revised: May 25, 2013

Date accepted: May 30, 2013

ABSTRACT

OBJECTIVE: To compare MutaGel HFE (commercial kit based on ARMS-PCR) with in-house designed polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) techniques for the diagnosis of C282Y and H63D point mutations in HFE-gene, and to recommend the feasibility of each method for individual laboratories according to their requirements.

METHODOLOGY: This quasi-experimental study was conducted at department of forensic and biomedical sciences, university of Lincoln UK from July 2006 to January 2007. Twenty-two samples were analyzed by two different molecular diagnostic techniques (ARMS & RFLP) for the same genetic disorder (C282Y and H63D mutation in HFE-gene). Blood samples were procured from healthy volunteers from different geographical areas. Two controls (one positive and one negative) were run with each batch. ARMS-PCR was carried out by using Mutagel HFE commercial kit by immunodiagnostik whereas PCR-RFLP was performed as per UK Haemochromatosis consortium directives.

RESULTS: Out of 22 samples for C282Y mutation; 20 showed normal genotype, one was heterozygous, none was homozygous and 1 showed sample contamination. For H63D mutation, 8 showed normal genotype, one was homozygous, 12 were heterozygous while one sample showed contamination.

There was no statistically significant difference in diagnostic sensitivity and specificity of both groups. Each method had its unique performance characteristics that could be utilized by different laboratories depending on their requirements.

CONCLUSION: Both the techniques were equally good for diagnosis of haemochromatosis but RFLP seemed more suitable for a laboratory with low workload whereas MutaGel HFE seemed more appropriate for high workload, less manpower and short turn-around time.

KEYWORDS: RFLP (Restriction Fragment Length Polymorphism), PCR (Polymerase Chain Reaction), ARMS (Amplification Refractory Mutation System), HFE gene mutation, Haemochromatosis.

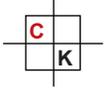
THIS ARTICLE MAY BE CITED AS: Haroon ZH, Javaid H, Williams DR, Kazmi SY. Comparison of MutaGel HFE and and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for the investigation of C282Y and H63D point mutation in HFE gene. *Khyber Med Univ J* 2013; 5(2): 61-65.

INTRODUCTION

Molecular biology has revolutionized modern medicine in understanding of the disease processes as well as assisting the clinicians in making diagnostic and therapeutic decisions. Diseases once thought to have monogenic Mendelian inheritance are now known to have polygenic complex patho-physiological mechanisms and confounding environmental factors. This understanding is solely due to the advancements in the molecular genetics.

Hereditary haemochromatosis is a prototype genetic disorder of iron overload that has an autosomal recessive mode of transmission.¹ Excessive iron absorbed from intestine is deposited in parenchyma of various organs especially liver, pancreas, heart, endocrine glands, skin etc. This causes fibrosis and irreversible damage to these tissues culminating in cirrhosis, hepato-cellular carcinoma, diabetes mellitus, hypogonadism, dilated cardiomyopathy, conduction defects of heart, skin pigmentation etc.^{2,3} Elevated serum ferritin and transferrin saturation is typically observed in these patients and these tests are currently used as screening test for hereditary haemochromatosis.^{4,5}

HFE gene is located on short arm of chromosome 6 and functions in regulating iron absorption from intestine.⁶ Loss of function of HFE gene products is associated with inappropriately increased iron absorption from intestine resulting in systemic disease. Two missense mu-



tations of HFE gene are recognized as C282Y and H63D.⁷ The C282Y mutation is found to be more penetrant of the two mutations.⁸ Although hereditary haemochromatosis is an autosomal recessive disorder, very rarely people may exhibit signs and symptoms of the disease with only one gene mutation called heterozygous.⁹

Heterozygous C282Y mutation has high prevalence among Caucasian population reaching approximately 10% while homozygous C282Y mutation is found in 3 to 5 subjects per thousand.⁴ Homozygosity for C282Y mutation or rarely compound heterozygosity for C282Y and H63D mutations may predispose an individual to phenotypic expression of hereditary haemochromatosis.¹⁰ However, the penetrance of C282Y homozygous mutation is extremely variable due either to environmental factors or overall genetic background of the individual.¹¹

Present study was conducted with the objectives to compare in-house designed PCR-RFLP with commercial kit MutaGel HFE based on ARMS technique for the diagnosis of C282Y and H63D point mutations in HFE gene, and to recommend the feasibility of each method for individual laboratories according to their own working requirements.

METHODOLOGY

This study was conducted at Department of Forensic and Biomedical sciences, University of Lincoln UK from July 2006 to January 2007. Twenty-two

samples were analyzed by two different molecular diagnostic techniques i.e. amplification refractory mutation system (ARMS) and restriction fragment length polymorphism (RFLP) for the same genetic disorder i.e. C282Y and H63D mutation in HFE gene. These blood samples were procured from healthy volunteers belonging to different geographical areas.

ARMS was carried out using MutaGel HFE commercial kit by immunodiagnostik. This kit contained different sets of lyophilized primers for analysis of C282Y and H63D mutation in HFE gene using ARMS. The primers sets were used in separate ARMS mixes for each mutation (in case of mutation C282Y, two ready to use tubes were provided; green colored PCR tube for wild type constellation of C282Y and blue colored PCR tube for mutation constellation of C282Y). The mixes contained both allele-specific primer pairs for the wild type and the mutation allele, as well as two consensus primers (the later ones generate the internal control fragment). All resulting amplification products were subsequently identified by agarose gel electrophoresis. Besides the internal control, at least one allele-specific DNA fragment was visible (or two as in case of heterozygous). The genotype was analyzed by interpretation of the specific DNA fragment pattern in the gel.

PCR-RFLP was performed as per UK Haemochromatosis consortium directives.¹² The procedure involved separate PCR amplification of regions within the

HFE gene containing the C282Y (exon 4; nt 845G→A) and H63D mutation. These were both positioned within restriction enzyme sites (for RsaI and MboI respectively). The PCR products were incubated with the appropriate enzyme overnight and the size of the resulting fragment determined by agarose gel electrophoresis. The PCR products had been designed to include an independent site for each restriction enzyme to provide internal control.

RESULTS

Twelve samples were of healthy volunteer males and ten were of healthy volunteer females. These people belong to different geographical areas as shown in table 1.

All these samples were analyzed by two different molecular diagnostics techniques i.e. RFLP and ARMS. The amplification products generated by both the techniques were analyzed by agarose gel electrophoresis shown in Figure 1, 2 and 3.

Out of 22 samples for C282Y mutation; 20 showed normal genotype, one was heterozygous and none was homozygous (Table 2). For H63D mutation, 8 showed normal genotype, one was homozygous and 12 were heterozygous (table 3). Results of both in-house designed PCR-RFLP and commercial kit MutaGel HFE based on ARMS technique were exactly alike.

TABLE I: SEX AND GEOGRAPHICAL DISTRIBUTION

| S.No | Number of samples (n=22) | Sex | Geographical origin |
|------|--------------------------|--------|---------------------|
| 1. | 07 | Female | English |
| 2. | 03 | Male | Walish |
| 3. | 03 | Male | Greek |
| 4. | 03` | Male | Spanish |
| 5. | 03 | Male | Pakistani |
| 6. | 03 | Female | Pakistani |

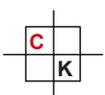


TABLE II: RESULTS OF C282Y ANALYSIS BY AMPLIFICATION REFRACTORY MUTATION SYSTEM (ARMS) AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

| Technique | Normal Genotype | Homozygous Mutant | Heterozygous Mutant | Sample Contamination |
|-----------|-----------------|-------------------|---------------------|----------------------|
| ARMS\$ | 20(90.9%) | Nil (0%) | 1 (4.5%) | 1 (4.5%) |
| RFLP* | 20(90.9%) | Nil (0%) | 1 (4.5%) | 1 (4.5%) |

\$amplification refractory mutation system, *restriction fragment length polymorphismwith the statement

TABLE III: RESULTS OF H63D ANALYSIS BY AMPLIFICATION REFRACTORY MUTATION SYSTEM (ARMS) AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

| Technique | Normal Genotype | Homozygous Mutant | Heterozygous Mutant | Sample Contamination |
|-----------|-----------------|-------------------|---------------------|----------------------|
| ARMS\$ | 8 (36.4%) | 1 (4.5%) | 12 (54.5%) | 1 (4.5%) |
| RFLP* | 8 (36.4%) | 1 (4.5%) | 12 (54.5%) | 1 (4.5%) |

\$amplification refractory mutation system, *restriction fragment length polymorphism

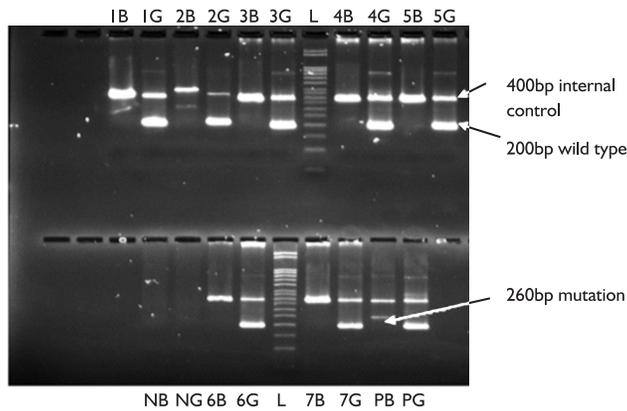


Fig 1: Amplification refractory mutation system - polymerase chain reaction (ARMS-PCR) analysis of C282Y mutation. B designates blue colored PCR tube and G for green colored PCR tubes (see text). Lanes N, P and L indicate negative controls, positive controls and DNA size ladder respectively.

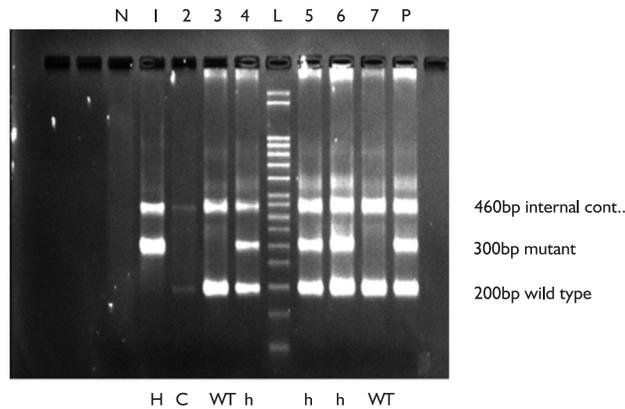


Fig 2: Amplification refractory mutation system - polymerase chain reaction (ARMS-PCR) analysis of H63D mutation. Lanes N, P and L indicates negative, positive PCR controls and DNA size ladder respectively. WT, wild type; h, heterozygous; H, homozygous; C, contaminated sample.

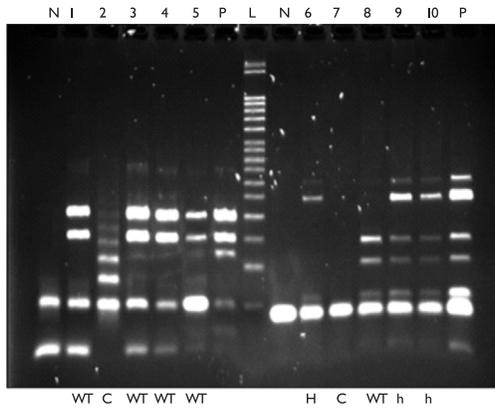


Fig 3: Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of C282Y and H63D mutations. Lanes N, P and L indicate negative controls, positive controls and DNA size ladder respectively. Lane 1,2,3,4,5 indicate C282Y; lanes 6,7,8,9,10 indicate H63D. Wt; wild-type, h; heterozygous, H; homozygous, C; contaminated sample.

DISCUSSION

Molecular biology has emerged as an essential device in routine diagnostic medicine. Nucleic acid based tests are increasingly being used and developed because these are sensitive, effective, and reliable in the diagnosis and management of genetic, hematologic, neoplastic, and infectious diseases.¹³ Hereditary haemochromatosis is one such genetic disorder where an early and accurate diagnosis is of paramount importance because timely initiation of phlebotomy may avert potentially lethal complications like cirrhosis and hepato-cellular carcinoma.¹⁴

Molecular diagnostics is an ever expanding field and many techniques are available for the diagnosis of genetic disorders. Different commercial kits are available as well as in-house designed primers are being used in different laboratories. Presently standardization of different assays is a problem. Different laboratories adopt different techniques for establishing diagnosis of the same genetic disorder depending on their own working requirements.

In our study we compared a commercial kit-MutaGel HFE based on ARMS-PCR technique with PCR-RFLP based on in-house designed primers. Both techniques were found to be equally sen-

sitive in detection of genetic mutations in HFE gene. Therefore, in our opinion, both techniques may be utilized for the molecular detection of point mutations in HFE gene for the genetic consultation of the families if the pathogenic mutation in a proband is detected. These results are in corroboration with a similar study conducted by Gunesacar et al. who compared ARMS and PCR-RFLP techniques for detection of MEFV gene exon point mutations in familial Mediterranean fever in a sample size of 90 and found both techniques equally sensitive and specific.¹⁵

In the present study, DNA isolation and agarose gel electrophoresis consumed same time and had similar cost for both the techniques. The main difference was in PCR procedure. For MutaGel HFE, PCR amplification required about 1 hour and 20 minutes. MutaGel HFE kit contained 24 tubes of lyophilized primers. For each lot of PCR, we needed to run a positive and a negative control, thus two tubes were consumed by the controls. It was, therefore, more economical to run samples in batches of 22.

For RFLP, the PCR required about 1 hour and 20 minutes followed by overnight enzyme digestion. This procedure was more time consuming due to enzyme digestion step. The total cost

of the raw material for this procedure (including primers, Taq polymerase, DNTP's, restriction enzymes and buffers etc) was considerably less than the cost of MutaGel HFE kit. But the number of samples that could be analyzed by this raw material was about three times that of MutaGel HFE single kit.

PCR-RFLP seemed more economical than MutaGel HFE (ARMS-PCR) but former was more time consuming. If, however, the labor cost (not considered in the present study) is also accounted for, then the cost of PCR-RFLP is likely to increase. However, we recommend another study with in-house designed ARMS primers and with large sample size to validate our results.

CONCLUSION

We conclude that both the techniques are equally good for the detection of C282Y and H63D mutations in HFE gene. The PCR-RFLP technique is more suitable for a laboratory with less workload whereas MutaGel HFE (commercial kit based on ARMS-PCR technique) seems more appropriate for laboratory with high workload, short turnaround time and less manpower.

ACKNOWLEDGMENT

We acknowledge the efforts of our

following participating investigators, for their contribution during the study project and writing of the manuscript

1: Dr. Marian Hill, Principal clinical scientist, Molecular diagnostics section, Department of clinical chemistry, Queens medical centre Nottingham U.K.

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AUTHOR'S CONTRIBUTION

Following authors have made substantial contributions to the manuscript as under:

ZHH: Conception and design, acquisition of data, analysis and interpretation of data, drafting the manuscript, final approval of the version to be published

HJ: critical revision, drafting the manuscript, final approval of the version to be published

DRW: Conception and design, acquisition of data, analysis and interpretation of data, final approval of the version to be published

SYK: critical revision, final approval of the version to be published

CONFLICT OF INTEREST

Author declares no conflict of interest

GRANT SUPPORT AND FINANCIAL DISCLOSURE

NIL

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