

Clinical Communications

A cost-effective algorithm for diagnosis of hereditary angioedema with normal C1 inhibitor: Applying molecular approach to clinical practice

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Clinical Implications

- Diagnosis of hereditary angioedema with normal C1 inhibitor is often a challenge to the clinician. The c.983C>A mutation in the gene encoding coagulation factor XII (*F12*) has been predominant as a cause of hereditary angioedema with normal C1-inhibitor among patients worldwide. A cost-effective algorithm may facilitate a definitive diagnosis by genetic analysis.

TO THE EDITOR:

Hereditary angioedema (HAE) is an autosomal-dominant disease characterized by recurrent edema of subcutaneous tissue, gastrointestinal tract, and upper airways.¹ Over a decade ago, HAE with normal C1 inhibitor (C1-INH) has been described, associated with mutations in *F12*, the gene encoding coagulation factor XII (FXII).² Subsequently, mutations in Plasminogen (*PLG*), Angiopoietin-1 (*ANGPT1*), and Kininogen 1 (*KNG1*) genes have been reported as cause of HAE with normal C1-INH.³⁻⁵ The newly described *KNG1* mutation could lead to a functionally active but aberrant bradykinin, which would undergo modified inactivation resulting in prolonged half-life.⁵ Four mutations in *F12*, all in exon 9, have been identified in patients with FXII-HAE: missense mutations c.983C>A (p.Thr328Lys) and c.983C>G (p.Thr328Arg), deletion c.971_1018+24del72, and duplication c.892_909dup.⁶ In a cohort of European patients with HAE with normal C1-INH, 26% of 265 individuals were carriers of *F12* mutations.⁶ A study from Brazil reported *F12* mutations in 134 of 195 patients with HAE with normal C1-INH, belonging to 42 families.⁷ In studies from different parts of the world, the c.983C>A (p.Thr328Lys) mutation has been identified in 519 of 551 (94.2%) patients belonging to 155 of 167 (92.8%) families (see Table E1 in this article's Online Repository at www.jaci-inpractice.org). We aimed to develop a cost-effective algorithm for the diagnosis of patients with HAE with normal C1-INH, using allelic discrimination to genotype the c.983C>A mutation as the initial step.

Patients with clinical suspicion of HAE with normal C1-INH, defined as those with symptoms suggestive of HAE, levels of C4 and quantitative and functional C1-INH within normal limits, and/or absence of mutations in *SERPING1*, were enrolled. A subgroup of patients participated in previous studies, comprising 15 families and 68 patients.^{7,8} Patients' selection, ethical aspects and methods for allelic discrimination, Sanger sequencing, and

calculation of costs are described in this article's Online Repository at www.jaci-inpractice.org.

One hundred eighty-four individuals, belonging to 51 families with index patients, underwent DNA analysis. Individuals were aged 2 months to 91 years, with a median age of 35 years, and 136 of 184 (73.9%) were female. Allelic discrimination was positive for the c.983C>A *F12* mutation in 24 of 51 (47%) index patients. On detection of the mutation in the index patient, respective family members underwent genetic analysis. Overall, 96 patients belonging to 24 families were positive for the c.983C>A *F12* mutation by allelic discrimination; all of them presented the c.983C>A *F12* mutation by Sanger sequencing (see Figure E1 in this article's Online Repository at www.jaci-inpractice.org). Twenty-seven index patients who were negative for the c.983C>A mutation as well as 61 individuals belonging to the 24 families described above who were negative on allelic discrimination were also negative for mutations in *F12* by Sanger sequencing. Kappa index was 1.00, indicating perfect concordance. Among the 96 patients bearing the c.983C>A mutation, 70 (72.9%) were symptomatic. The proportion of symptomatic male carriers (56%) was higher as compared with that reported among European patients (4%-37.5%).^{7,8}

None of the 27 index patients negative for the c.983C>A mutation presented mutations in *PLG* or *ANGPT1* genes. Patients with family history and no causative genetic variants identified were characterized as HAE with normal C1-INH presenting as unknown HAE (*n* = 11); those with angioedema and normal C1-INH without family history were diagnosed with idiopathic nonhistaminergic acquired angioedema (*n* = 16).

Costs and time for performing allelic discrimination were US \$91.08 and US \$194.37, and 6 and 7.5 hours, for 1 and 10 samples, respectively. Values for DNA sequencing by the Sanger method were US \$496.69 and US \$776.28, and 38.5 and 43.5 hours, for 1 and 10 samples, respectively (Figure 1, see Table E2 in this article's Online Repository at www.jaci-inpractice.org).

We identified the c.983C>A mutation in 100% of our patients with FXII-HAE. Allelic discrimination was more cost-effective, with reduction of 82% in costs and decrease of 84% in performance time, compared with Sanger sequencing. Additional cost reduction and gain in time could be obtained by testing multiple samples. Reasons for these differences include fewer steps, fewer reagents used, and shorter labor time. Therefore, we propose that allelic discrimination for the c.983C>A mutation in *F12* could be used as an initial diagnostic test for HAE with normal C1-INH. No other mutations in *F12* and no previously reported mutations in *PLG* and *ANGPT1* were identified in patients negative for the c.983C>A mutation. These results led us to suggest an algorithm for the diagnosis of HAE with normal C1-INH, which could be useful in areas where the c.983C>A mutation in *F12* is predominant (Figure 2).

Genetic analysis revealed the diagnosis of FXII-HAE in 96 individuals belonging to 24 families with index patients with clinical suspicion of the disease, including 26 asymptomatic patients who otherwise would remain undiagnosed. Medications for acute attacks licensed for patients with C1-INH-HAE including icatibant, ecallantide, and plasma-derived C1-INH concentrate have been successfully used in patients with HAE with normal C1-INH.⁹ In addition, patients with FXII-HAE

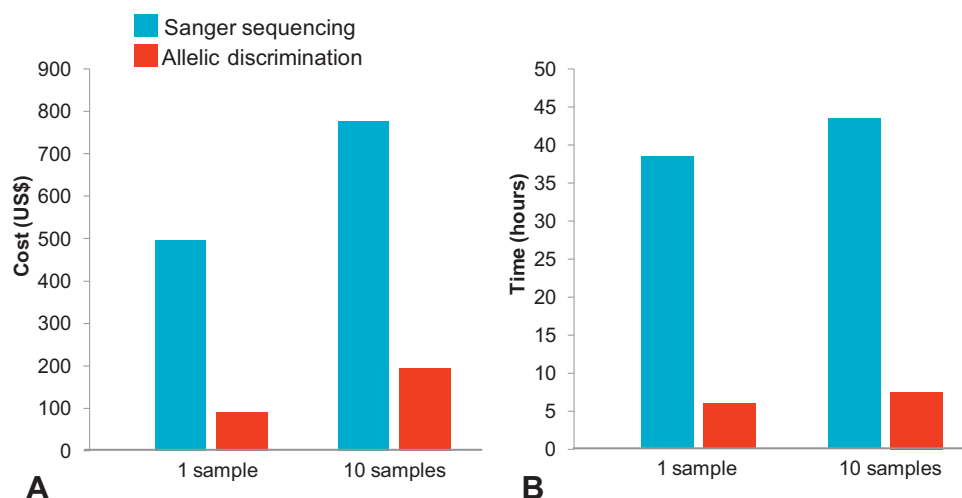


FIGURE 1. Comparison of (A) total costs and (B) time, including blood collection and DNA preparation, for performing Sanger sequencing and allelic discrimination for the c.983C>A mutation in *F12*, for 1 and 10 samples.

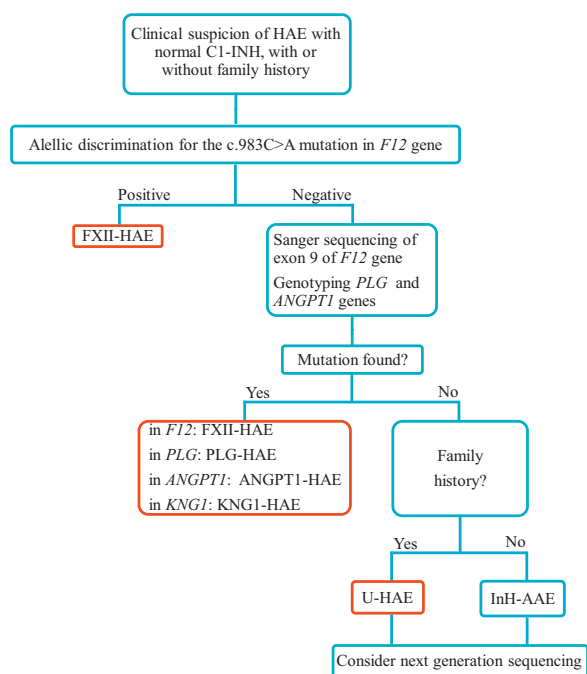


FIGURE 2. Algorithm to investigate HAE with normal C1-INH. *ANGPT1-HAE*, HAE with mutation in the gene encoding Angiopoietin-1 (*ANGPT1*); *FXII-HAE*, HAE with mutation in the gene encoding coagulation factor XII (*F12*); *InH-AAE*, idiopathic nonhistaminergic acquired angioedema; *PLG-HAE*, HAE with mutation in the gene encoding Plasminogen (*PLG*); *U-HAE*, unknown HAE. HAE with normal C1-INH types are circled in red.

show a good response to tranexamic acid for prophylaxis of HAE attacks.⁹ Therefore, patients diagnosed early would benefit from prompt treatment and preventive strategies and would be spared of diagnostic uncertainties.

In conclusion, the allelic discrimination method was developed for the detection of c.983C>A mutation in *F12*. Sanger

sequencing including sequence of exon 9 and intronic adjacent regions of *F12* would be indicated as the next step in patients with negative allelic discrimination results for the c.983C>A mutation. Allelic discrimination could be developed for simultaneous detection of missense mutations c.983C>G in *F12*,² c.988A>G in *PLG*,³ c.807G>T in *ANGPT1*,⁴ and c.1136T>A in *KNG1*,⁵ in a multiplex assay format. Patients negative to known mutations in *F12*, *PLG*, *ANGPT1*, and *KNG1* genes and angioedema with normal C1-INH could benefit from further investigation of pathogenic genetic variants by next-generation sequencing. Alternatively, in areas where *F12* mutations are rare, whole-exome sequence could be considered as the diagnostic method of choice. Uncertain diagnosis often leads to patients' anxiety, because of the bothersome and sometimes life-threatening nature of the attacks. Therefore, despite higher costs of next-generation sequencing approaches (see Table E2), precise diagnosis would be worth pursuing, providing grounds for adequate treatment and genetic counseling. Further studies in other areas need to be conducted to establish whether allelic discrimination would have a lower cost and provide faster results.

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METHODS

Patients' selection and ethical aspects

Patients attended the Allergy Clinics of the Clinical Hospital of Ribeirão Preto Medical School or had biological material sent to our laboratory for genetic investigation. The study was approved by the Ethics Committee of our institution (protocol no. 2344/2017), and all participants or their legal guardians signed written informed consent.

Allelic discrimination and Sanger sequencing

Genomic DNA was extracted from whole blood or oral mucosa cells using the DNA Wizard Genomic DNA Purification Kit (Promega, Madison, Wis). Allelic discriminations were carried out by real-time PCR using chromophore probes VIC (554 $\lambda_{\text{max}}/\text{nm}$) designed for the wild-type allele and FAM (518 $\lambda_{\text{max}}/\text{nm}$) designed for the allele with the c.983C>A mutation. Primers were F 5'-CTAGGCTTCATGTCCCACTCAT-3' and R 5'-CCCCCACTTCCTAACCTC-3', and reporters were Reporter 1 VIC TCAGCCCACGACCCG and Reporter 2 FAM CAGCCCAAGACCCG. Real-time PCR was performed in 10.5 μL volume containing 15 ng genomic DNA, TaqMan Genotyping Master Mix, and TaqMan Probe (Thermo Fisher Scientific, Waltham, Mass). Samples were processed in the Applied Biosystems 7500 Real Time Thermal Cycler (Applied Biosystems, Foster City, Calif) using the following steps: 1 minute at 60°C, 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C followed by 1 minute at 60°C, and 60° for 1 minute. Results were analyzed by ABI 7500 SDS software (Thermo Fisher Scientific, Waltham, MA).

Sequencing of exon 9 of *F12* gene by Sanger technique was performed using Applied Biosystems 3500 Genetic Analyzer sequencer (Applied Biosystems). PCR was performed to generate a 395 base-pair fragment, comprising a region of exon 9 and adjacent intronic regions of *F12*, which allows for the detection of all previously described mutations in *F12* associated with FXII-HAE.^{E1} PCR product was purified by ExoSAP-IT (Thermo Fisher Scientific) and sequenced.

To investigate previously described mutations in *PLG* and *ANGPT1* genes, the following primers were used: *PLG* primers F 5'-CTT AGT TTT AGT TAC TGT AGG AAC GCA GG-3' and R 5'-CAG GCT TTC TGA CCA CAA TAG C-3' to amplify a 494 base-pair fragment^{E2}; *ANGPT1* primers F 5'-GTTGACAACTGGATTCCTGTGTG-3' and R 5'-GTTGACAACTGGATTCCTGTGTG-3' to amplify a 446 base-pair fragment. *ANGPT1* primers were designed using the Gene Runner Software v.3.05 (Hastings Software, Inc, Hastings, NY). PCR was carried out in 25 μL volume containing 100 ng of genomic DNA, 0.5 μL of each primer 10 mM (IDT, Coralville, Iowa), DNA polymerase Taq Platinum (Thermo Fisher Scientific) using 1.0 μL of MgCl_2 for *PLG* and 0.5 μL of MgCl_2 for *ANGPT1*, and 1.0 μL dNTP 5 mM (Thermo Fisher Scientific). Samples were processed in Eppendorf Mastercycle Pro (Eppendorf, Hamburg, Germany) using the following steps: 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 2 minutes. PCR products were purified with ExoSAP-IT (Thermo Fisher Scientific) and sequenced by Sanger technique using Applied Biosystems 3500 Genetic Analyzer sequencer (Applied Biosystems).

Evaluation of costs

Direct costs included costs of reagents and consumables, direct labor, sequencing service, energy, and maintenance of equipment directly involved in performing the tests, which were calculated on the basis of Brazilian guidelines.^{E3} Calculation was based on Brazilian values in Reais and converted to US dollars at the rate of US \$ 1.00 = R\$ 3.15. Costs for the collection of blood were provided by the Clinical Hospital of Ribeirão Preto Medical School financial management office. Attempts to evaluate indirect costs, including building maintenance and security, water, telephone, internet, warehouse materials, administrative services, and equipment not directly involved in the tests, were hampered by the fact that it was not possible to measure them accurately at each process.

TABLE E1. Description of mutations in the *F12* gene reported among patients with HAE with normal C1-INH

Reference	Origin	Type of mutation in the <i>F12</i> gene	No. of families with mutation	No. of patients with mutation*
Veronez et al, ^{E1} 2017	Brazil	c.983C>A (p.Thr328Lys)	41 of 42 (97.6%)	132
		c.971_1018+24del 72	1 of 42 (2.4%)	2
Bouillet et al, ^{E4} 2017	France	c.983C>A (p.Thr328Lys)	1	2
Gallais Sérézal et al, ^{E5} 2016	Sweden	c.983C>A (p.Thr328Lys)	3	3
Piñero-Saavedra et al, ^{E6} 2016	Spain	c.983C>A (p.Thr328Lys)	9 of 9 (100%)	35
Bork et al, ^{E7} 2015	Germany/Europe	c.983C>A (p.Thr328Lys)	19 of 23 (82.6%)	92
		c.983C>G (p.Thr328Arg)	2 of 23 (8.7%)	5
		c.971_1018+24del 72	2 of 23 (8.7%)	7
Charignon et al, ^{E8} 2014	France, Spain, Algeria, Germany, Switzerland	c.983C>A (p.Thr328Lys)	40 of 40 (100%)	118
Bork et al, ^{E9} 2014	Turkey	c.971_1018+24del 72	2	7
Kiss et al, ^{E10} 2013	White	c.892_909dup	1	5
Marcos et al, ^{E11} 2012	Spain	c.983C>A (p.Thr328Lys)	13 of 13 (100%)	29
Baeza et al, ^{E12} 2011	Morocco/Arab	c.983C>A (p.Thr328Lys)	1	4
Vitrat-Hincky et al, ^{E13} 2010	France	Mutation not reported	3	6
Picone et al, ^{E14} 2010	France	c.983C>A (p.Thr328Lys)	2	3
Bork et al, ^{E15} 2009	Germany	c.983C>A (p.Thr328Lys)	11 of 13 (85%)	32
		c.983C>G (p.Thr328Arg)	2 of 13 (15%)	3
Hentges et al, ^{E16} 2009	Luxemburg	c.983C>A (p.Thr328Lys)	1	1
Nagy et al, ^{E17} 2009	UK/Italy	c.983C>A (p.Thr328Lys)	1	5
Duan et al, ^{E18} 2009	Italy	c.983C>A (p.Thr328Lys)	1	9
Prieto et al, ^{E19} 2009	Spain	c.983C>A (p.Thr328Lys)	1	4
Bell et al, ^{E20} 2008	Australia	c.983C>A (p.Thr328Lys)	1	1
Martin et al, ^{E21} 2007	France	c.983C>A (p.Thr328Lys)	1	7
Bouillet et al, ^{E22} 2007	France	c.983C>A (p.Thr328Lys)	1	2
Cichon et al, ^{E23} 2006	Germany/France	c.983C>A (p.Thr328Lys)	1	6
Dewald and Bork, ^{E24} 2006	Germany/Europe	c.983C>A (p.Thr328Lys)	5 of 6 (83.3%)	27
		c.983C>G (p.Thr328Arg)	1 of 6 (16.7%)	4
Combined studies		c.983C>A (p.Thr328Lys)	155 of 167 (92.8%)	519 of 551 (94.2%)

*May include asymptomatic carriers.

TABLE E2. Costs and time for performing Sanger sequencing and allelic discrimination

Parameter	Sanger sequencing*		Allelic discrimination†	
	1 sample	10 samples	1 sample	10 samples
Blood collection	1.19	11.90	1.19	11.90
Preparation of DNA‡	34.58	89.22	34.58	89.22
Reagents and consumables	10.00	47.96	9.28	32.13
Technical workforce	213.94	237.71	41.60	47.54
Equipment cost§	0.23	2.34	3.40	11.03
Equipment maintenance	0.05	0.47	0.68	2.20
Power consumption	1.48	1.48	0.35	0.35
Sequencing	4.76	47.62	NA	NA
Repeated analysis	230.46	337.58	NA	NA
Total (US \$)¶	496.69	776.28	91.08	194.37
Time (h)	38.5	43.5	6.0	7.5

NA, Not applicable.

*Sanger sequencing steps were conventional PCR, electrophoresis, purification of PCR products, sequencing reactions, precipitation, sequencing, and analysis of results.

†Allelic discrimination steps were real-time PCR and analysis of results by VIC and FAM fluorescence.

‡Preparations of DNA steps were extraction, quantification, and dilution of DNA.

§Equipment cost was estimated on the basis of actual cost and durability of the equipment, and the predicted time for performing experiments.

||Repeated analysis was performed when chromatograms did not fulfill quality requirements to allow accurate readings, requiring that all sequencing steps be repeated. In addition, the presence of mutation by Sanger sequencing was confirmed by repeating sequencing steps. For the purpose of calculation of costs, it was assumed that all sequence reactions were performed in duplicate.

¶For next-generation sequence approaches, estimates in US dollars ranged from \$555 to \$5,169 for a single whole-exome sequence (WES) test and from \$1,906 to \$24,810 for a single whole-genome sequence (WGS) test, according to a recent systematic review of the literature, based on published data from different parts of the world: the United States, the United Kingdom, Canada, Australia, the Netherlands, and France, in various genetic conditions, most commonly neurological or neurodevelopmental disorders.^{E25} Similar figures were encountered in Brazil, with \$1,894 for the WES test and \$3,834-\$5,225 for the WGS test.

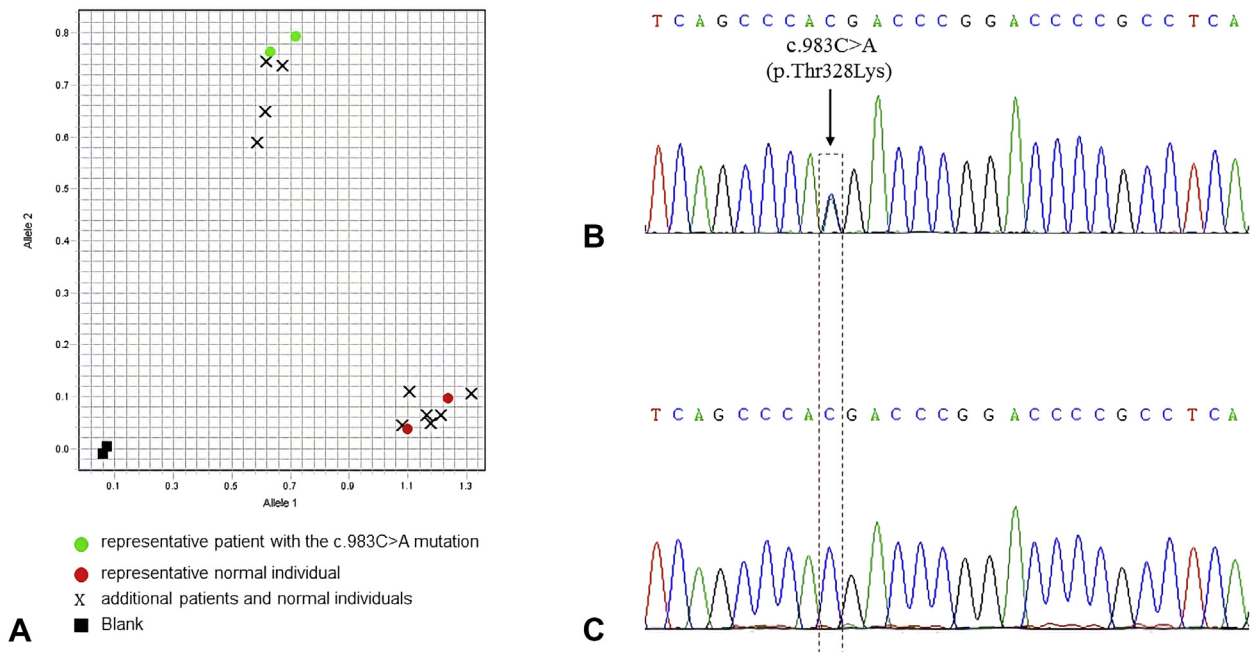


FIGURE E1. Genotyping of variant c.983C>A in exon 9 of *F12* gene. **(A)** Representative TaqMan assay for genotyping the c.983C>A variant (heterozygous) in 7 individuals, highlighting 1 patient with the c.983C>A variant (*green*) and 1 individual with wild-type allele (*red*), with experiments performed in duplicate. Sequencing by the Sanger method in **(B)** a patient with the c.983C>A mutation (*arrow*) and in **(C)** a normal individual.

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