Field evaluation of the GeneXpert system for detection of thrombophilia associated mutations: a six-month experience in a small laboratory

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ABSTRACT

Factor V Leiden G1691A mutation (FVL) and prothrombin G20210A mutation (GPro) are the most common inherited mutations associated with thrombophilia. GeneXpert HemosIL Factor II and Factor V assay is a fully automated assay that is able, in less than 35 min, to allow simultaneous detection of FVL and GPro. Test format, based upon single test cartridge, was designed to minimize waste and to permit daily analytical sessions. In this study we evaluated the performance of GeneXpert system in detection of FVL and GPro mutations. 211 consecutive patients, enrolled from March to August 2011, were studied. All samples were evaluated by using the GeneXpert system in comparison with Roche Light Cycler assay. By using both assays, 51 FVL heterozygous, 3 FVL homozygous, 1 GPro homozygous, 10 GPro heterozygous, 5 combined FVL-GPro heterozygous and 141 normal subjects were identified, with a 100% concordance between the two assays. During six months we observed 15 invalid sample results using GeneXpert (7.1%) that were retested after dilution. Consequently, the tests/results ratio was 1.07. In our experience, the assay was therefore affordable and characterized by a good rate between number of carried out tests and released results.

INTRODUCTION

Venous thrombosis is the third most common vascular disease after ischemic heart disease and stroke. It is common in whites, affecting 1 in 1000 individuals every year, and is strongly associated with life-threatening pulmonary embolism. In addition to aging and circumstantial predisposing factors (e.g., surgery, pregnancy or immobilization), genetic risk factors, due to molecular abnormalities of components of the coagulation pathway, leading to hypercoagulability and, in turn, to thrombophilia, have been found in subjects, who suffered thromboembolic disease. In subjects from different ethnic groups, a common mutation [a G-to-A transition at nucleotide 1691 within the factor V gene: FV Leiden (FVL)], leading to resistance to activated protein C (aPC), has been found in up to 20% of cases of patients with deep venous thrombosis (1, 2). Another common mutation, a G-to-A transition at nucleotide position 20210 within the prothrombin gene locus (GPro), has been described, the A allele carriership being associated with a higher risk of venous thrombosis and accounting for up to 14% of cases of deep venous thrombosis (3, 4).

FVL is an autosomal dominant condition that exhibits incomplete dominance and results in a factor V variant that cannot be as easily degraded by aPC. The gene coding the protein is referred to as F5. FVL is characterized by a single nucleotide polymorphism in exon 10, in position 1691 with a G to A substitution (G1691A); it also predicts a single amino acid replacement [arginine to glutamine (R506Q)] at one of the three aPC cleavage sites in the factor Va. Since this amino acid is normally the cleavage site for aPC, the mutation prevents efficient inactivation of factor V. When factor V remains active, it facilitates overproduction of thrombin leading to excess fibrin generation and excess clotting.

Evaluation for FVL can begin with a test for aPC resistance. Indeed, in ~95% of cases the aPC resistance is due to a FVL mutation. If resistance is present, then a genetic test for FVL gene mutation has to be performed, both to confirm the diagnosis and to determine whether the subject is heterozygous or homozygous for the mutation (5, 6).

Prothrombin is the precursor of thrombin in the coagulation cascade; thrombin is required to convert fibrinogen into fibrin, which is the primary goal of the coagulation cascade. As said, the GPro is caused by a G to A transition in the 3'-untranslated region of the prothrombin (factor II) gene at position 20210. This condition is associated with a mild increase in plasma

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prothrombin concentrations and an increased risk of venous thrombosis. Diagnosis of GPro mutation is made through specific genetic testing, which can also reveal whether the patient is heterozygous or homozygous for the condition. Although prothrombin levels are usually moderately elevated in subjects with this mutation, these results are not clinically useful in identifying the mutation (7, 8).

In this study, we evaluated the GeneXpert system for combined detection of FVL and GPro in comparison with the Roche Light Cycler assay, routinely used in our laboratory.

MATERIALS AND METHODS

Study design

This study was performed in a little clinical laboratory (about 1,500,000 tests/year) located in a non teaching 300-bed general hospital. Between March and August 2011, 211 consecutive patients, referred to our laboratory for evaluating thrombophilia risk factors, were studied. These patients were evaluated in duplicate for FVL and GPro by using GeneXpert and Roche Light Cycler assays. After approval by the local Ethic Committee, this study was carried out according to the principles of the Helsinki declaration; informed consent was obtained from all subjects.

For the study, blood samples were collected into vacuum tubes (Becton Dickinson) containing 0.129 M trisodium citrate and stored at +4 °C up to 6 h, before test execution.

Methods

Roche Light Cycler assay

DNA samples were isolated from whole blood with the aid of a MagNa Pure LC DNA Isolation kit I, using a MagNA Pure LC automated DNA isolation instrument (Roche Molecular Biochemicals). DNA samples were stored at -20 °C until the mutations were investigated. FVL and GPro mutations were detected with the Light Cycler-Factor V Leiden and Light Cycler-Prothrombin 20210GA mutation detection kits (Roche Molecular Biochemicals), respectively. All mutation-related gene regions were amplified in 20 µL polymerase chain reaction (PCR) capillary tubes. After preparation of the master mixture, 18 µL of the reaction mixture and 2 µL (approximately 40 ng) of genomic DNA or control template were added to each Light Cycler capillary tube. For negative control, PCR grade water was added instead of template. The capillary tubes were sealed and briefly centrifuged in a microcentrifuge and then placed into the Light Cycler carousel. The PCR products were detected using 3'-fluorescein (FLU)-labelled and 5'-Red 640-labelled probes. When both probes hybridize in close proximity, fluorescence resonance energy transfer (FRET) occurs, producing a specific fluorescence emission of Light Cycler-Red as a result of FLU excitation. The fluorescence intensity depends on the amount of specific PCR products. Amplification per cycle can be monitored with the Light Cycler instrument. At the end of the amplification process, the Light Cycler instrument increases the temperature and the fluorescence obtained is plotted against the temperature. The mutations are then identified by their characteristic curves. Total assay time is approximately 40 min (9, 10).

GeneXpert HemosIL Factor II and Factor V assay

The GeneXpert is a software-driven cartridge processor with an integrated thermal cycler with a fluorescence-based detection system that adopt single-use disposable cartridges to obtain nucleic acid isolation from whole blood and perform a quantitative PCR. GeneXpert cartridge consists of multiple chambers for complete automation of nucleic acid extraction from whole blood, PCR reaction, products revelation and quantification. Cartridges contain chambers for sample introduction, lysis buffer, purification and elution buffers and all real-time PCR reagents and enzymes in two freeze-dried beads: bead 1 for polymerase and nucleotides, bead 2 for primers and probes. Moreover, all sample-processing wastes are retained within the cartridge. The cartridge also has an attached PCR tube with fluidic connections to the reagents in the cartridge chambers; after introduction into the analyzer, this PCR tube is surrounded by heating/cooling plates and by optical blocks that enable amplification and real-time, fluorescence-based PCR product detection. At the center of the cartridge is a syringe barrel that has a dry interface (to minimize potential for contamination) with the GeneXpert device through a syringe plunger, thereby allowing the movement of fluids within the cartridge and between the PCR tube and the cartridge (11, 12). Fluid movement within the cartridge is controlled by a rotary valve. The valve body contains a cavity in which nucleic acid purification beads are located. Within the cavity there are inlet and outlet ports that provide for fluid movement over the purification beads. These beads are retained within the cavity by screens that cover the two ports on the back of the valve body and a flat cap on the front of the valve body. For nucleic acid capture, a whole blood lysate solution from one reagent chamber is flowed through the beads within the valve body (13, 14). The bound nucleic acid is then washed, eluted, mixed with PCR reagents, and dispensed into the PCR tube for amplification and real-time detection by scorpion technique. Scorpion primers are bi-functional molecules in which a primer is covalently linked to the probe. The molecules also contain a fluorophore and a quencher. In the absence of the target, the quencher nearly absorbs the fluorescence emitted by the fluorophore. During the scorpion PCR reaction, in the presence of the target, the fluorophore and the quencher separate, which leads to an increase in the fluorescence emitted. The fluorescence can be detected and measured in the reaction tube (15-17). Examples of revelation curves obtained are reported in Figure 1.

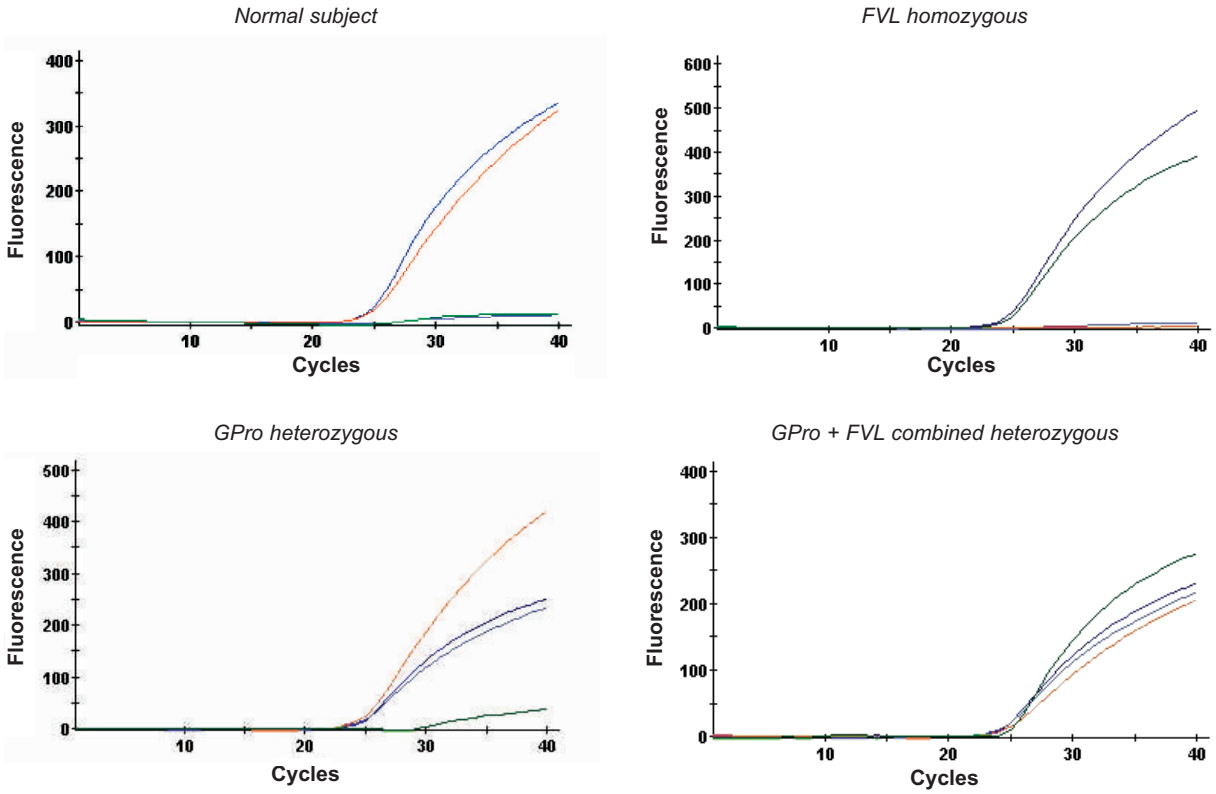

Figure 1
Amplification curves obtained using the GeneXpert HemosIL Factor II and Factor V assay in different types of subjects.

RESULTS

All the samples evaluated in this study were obtained from patients of Italian ancestry, with age from 14 to 69 years (mean, 49 years); 136 (64.5%) were females and 75 (35.5%) were males. Among the 211 considered subjects, by using both Light Cycler and GeneXpert assays, 51 FVL heterozygous, 3 FVL homozygous, 10 GPro heterozygous, 1 GPro homozygous, 5 combined FVL-GPro heterozygous and 141 normal subjects were identified. We observed a 100% concordance between results obtained by using Roche Light Cycler assay and GeneXpert HemosIL Factor II and Factor V.

During the 6-month period of evaluation, we observed 15 invalid sample results (7.1%). As reported in Figure 2, the prevalence of invalid samples decreased from the first to the sixth month of analytical activity, in correspondence with an improvement of operator experience. All these samples were retested after 1:5 sample dilution, giving a valid result. So, to produce 211 results we performed 226 tests, with a tests/results ratio of 1.07.

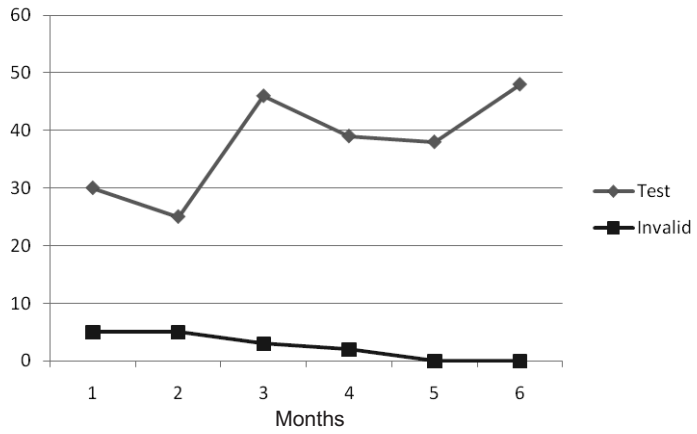

Figure 2
Number of performed tests and number of invalid tests using the GeneXpert system during six months.

DISCUSSION

FVL mutation is the most important genetic risk factor for venous thromboembolism and the mutation in the 3'-untranslated region of the prothrombin gene seems to be another mild risk factor for thrombotic events. It has been clearly shown that FVL is frequently co-inherited with the GPro variant (18). The association of the two prothrombotic alleles indicate that the prothrombin variant is an additional risk factor for venous thromboembolism and might contribute to thrombotic manifestations (19).

Chioggia is a town of about 60,000 inhabitants, built in a group of islands in the southern part of the Venice lagoon; for logistic reason, there is actually only a street connecting Chioggia with the mainland and, until the end of XIX century, the town remained quite secluded from the surrounding areas. So that, as previously reported, we observed a high prevalence of FVL in patients studied to detect thrombophilia risk factors after thrombosis, even in the open population (20-23). Therefore, a rapid, simple and relatively cheap test for the simultaneous detection of the point mutations for FVL and GPro was considered of great utility in our laboratory practice. Various different methods for the simultaneous detection were described in literature. All these methods required DNA extraction followed by amplification reaction and, often, further analysis of the amplification products, e.g., enzyme digestion (9, 10, 24, 25).

We reported here our field experience by using a fast and simple test for simultaneous detection of FVL and GPro. In our evaluation, GeneXpert assay for combined detection of FVL and GPro showed a full concordance with Roche Light Cycler assay. Another advantage of the simultaneous FVL and GPro assay was the reduction in costs and labour by 50%, because patients with a mutated allele for factor V and/or prothrombin variant are identified in a single step.

The GeneXpert system represents an important new development in the field of molecular diagnostics. It automates all steps of nucleic acid amplification in a disposable, microfluidic cartridge. It is a moderately complex test, but is simple enough to be reliably performed by individuals without a specific background in nucleic acid diagnostics. Samples and buffers are the only components to be added to the cartridge by the user. The device has independently controlled and operated analysis modules that facilitate testing of individual samples in a random-access mode. The test incorporates an internal control, which ensures that the entire test system is functioning properly, and a probe check control step, performed before PCR, to verify reagent rehydration, probe integrity, and PCR tube filling in the cartridge. So, by using the GeneXpert HemosIL Factor II and Factor V assay, it is possible to perform genetic tests in complete automation from the addition of whole blood and reagents to the cartridge to final results, without the need of further analytical phase, e.g., DNA extraction, or the need of special location for molecular biology assays. The assay format was studied to minimize waste and to permit single test execution. The system is uniquely suited for clinical applications of molecular diagnostics when on-demand testing and rapid-result capability are needed.

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