academicJournals

Vol. 12(19), pp. 2706-2710, 8 May, 2013
DOI: 10.5897/AJB2013.12043
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Technological and cost comparison of cytochrome P450 2B6 (516G>T) genotyping methods in routine clinical practice

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Accepted 17 April, 2013

Pharmacogenetics requires robust and affordable tests to determine genetic variability. This study compares three genotyping methods: gene re-sequencing, real time polymerase chain reaction (PCR) allelic discrimination and PCR-RFLP for the detection of a genetic variation (516G>T) in the gene which codes for the enzyme, CYP2B6, the main enzyme in the metabolic pathway of the antiretroviral drug, efavirenz. The CYP2B6 (516G>T) variant has reduced metabolic capacity. Twenty (20) samples obtained from human immunodeficiency virus acquired immunodeficiency syndrome (HIV/AIDS) positive patients on an efavirenz containing regimen were used to establish whether these methods produce the same CYP2B6 genotype results on the same samples. Results were directly compared for concordance and revealed a 100% correlation with all three methods. Comparison for cost of equipment and reagents required for each method revealed an order of: sequencing > real time-PCR > PCR-RFLP. This study demonstrates the reproducibility of these three methods and provides an opportunity for the clinical applicability in routine clinical practice.

Key words: Polymorphism, drug response, efavirenz, Zimbabwe.

INTRODUCTION

Pharmacogenetics, the effect of genetic variability on drug response with respect to efficacy and safety of drugs, is increasingly transforming the practice of medicine from one treatment/dose fits all to personalised treatment (Goldstein et al., 2003; O'Kane et al., 2003; Ingelman-Sundberg, 2008). Variation in genes coding for drug target proteins (receptors, transporters and enzymes) have been shown to affect the pharmacodynamics and pharmacokinetics of some drugs resulting in the responder and non-responder patient phenotypes which in turn affects drug efficacy and safety (Sheffield and Phillimore, 2009; Tozzi, 2010). In recognition of these developments, Food and Drug Administration (FDA), World Health Organisation (WHO), European Medicines Agency (EMA), the Pharmaceutical industry and other regulatory authorities have come up with guidelines on the conduct of pharmacogenetic studies, the validation of pharmacogenetic tests, and the clinical practice of personalised medicine (Kirchheiner et al., 2005). Over 70 drugs in the market now carry pharmacogenetics information and/or recommendations to take into consideration their use and over 20 Pharmacogenetic tests have been approved or are at various levels of approval (http://www.fda.gov/drugs/scienceresearch/researcharea

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s/pharmacogenetics/ucm083378.htm).

Pharmacogenetic findings that have been developed for clinical solutions have mainly been on genes that affect the pharmacokinetics of drugs (Sheffield and Phillimore, 2009). The importance of pharmacogenetics in the treatment of HIV/AIDS was first highlighted by the discovery of HLA-B*5701 single nucleotide polymorphism (SNP) as a high predictive biomarker for potentially fatal skin hypersensivity reaction to abacavir (Hetherington et al., 2002; Martin et al., 2004). A genetic test for this SNP was subsequently developed and approved by FDA in 2008 (http://www.fda.gov/drugs/scienceresearch/researchareas/pharmacogenetics/ucm083378.htm). The metabolism and disposition of efavirenz has been demonstrated to be mainly by CYP2B6 (Ward et al, 2003; Tsuchiya et al., 2004; Rotger, 2007). Several SNPs of this enzyme have been shown to determine exposure levels of efavirenz with CYP2B6 G516T and CYP2B6 T983C being the most significant (Rotger et al., 2005). The frequency of CYP2B6 G516T has been shown to be very high in people of African origin as compared to Caucasian and Oriental populations (Matimba et al., 2008). Pharmacokinetic simulation studies have led to the derivation of a pharmacogenetic based dosing algorithm that takes the CYP2B6G516T genotype into account. Patients homozygous for this variant have been shown to need only a third (200 mg) of the standard dose (600 mg) to attain safe and efficacious levels (Nyakutira et al., 2008). Ongoing studies are evaluating this dosing algorithm in a greater number of patients. Given the potential clinical utility of this dosing algorithm, there is need for a pharmacogenetics diagnostic test for CYP2B6 polymorphism.

A number of methods for determining the CYP2B6G516T polymorphism have been published; the PCR-RFLP method (Rotger et al., 2005), the real-time PCR allelic discrimination method (Applied Biosystems, Foster City, CA) and the direct sequencing method (Mardis, 2008). In this study, we have compared the performance of these three methods on a set of 20 samples. The rationale for this being the need to demonstrate the reproducibility of these methods on the same samples, thus guide interested diagnostic laboratories on methods to adopt for clinical diagnostics.

The comparison was also done with a view to guide choice of method to invest in by interested parties whilst considering the key elements of a pharmacogenetic test for successful clinical application. The key elements assessed were experimental/technical robustness, accessibility (availability of laboratory facilities that can offer test in a timely manner) and affordability (a favourable cost-benefit ratio in resource limited settings).

MATERIALS AND METHODS

A total of 20 samples from HIV/AIDS patients from Wilkins Hospital in Harare receiving efavirenz (600 mg once daily) in combination with two nucleoside analogue inhibitors, stavudine and lamivudine, were analyzed. Ethical approval for this study was obtained from the Medical Research Council of Zimbabwe. Whole blood was collected in EDTA tubes from each patient. Total genomic DNA was isolated using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

PCR-RFLP

Genotyping was done using a PCR-RFLP method according to Rotger et al. (2005). The forward (5'-GTCTGCCCATCTATAAAC-3') and reverse (5'-CTGATTCTTCACATGTCTGCG-3') primers were used to generate a 526 bp product. A no DNA control was included in all the reactions to check for contamination. Briefly, PCR was performed using the GeneAmp PCR system 9700 in a total reaction volume of 15 µL with 5 to 10 ng of genomic DNA and Taq DNA polymerase (Inqaba Biotech, South Africa). PCR conditions consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 20 s, and extension at 72°C for 1 min, and a final extension at 72°C for 60 s. The PCR product was digested with *BsrI* (New England Biolabs, USA) for 2 h at 65°C according to the manufacturer's instructions.

TaqMan real-time PCR allelic discrimination

Genotyping was carried out by TaqMan allelic discrimination with fluorogenic 5´ nuclease assays on an ABI 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). SNPs were analyzed using the following validated TaqMan Genotyping Assay purchased from Applied Biosystems: rs3745274, Assay ID C___7817765_60 according to the manufacturer's instructions. Amplification conditions consisted of an initial hold cycle at 94°C for 10 min, followed by 50 cycles of denaturation at 92°C for 15 s, annealing and extension at 90°C for 1 min. A no DNA control was included in all the reactions to check for contamination.

Re-sequencing

Briefly, PCR was performed using the GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) in a total reaction volume of 20 µl with 10 ng of genomic DNA and Ex Taq DNA polymerase (Takara Bio, Shiga, Japan). PCR conditions consisted of an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturetion at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 5 min (Maimbo et al., 2012). Sequencing of the purified DNA sample was carried out using the 3730 x l DNA Analyzer (Applied Biosystems). Sequences were analyzed using the Sequencer software 4.8.

RESULTS AND DISCUSSION

Comparison of results obtained using the three methods revealed a 100% correlation. The following genotypes were observed, four homozygous wild type (516 GG), thirteen heterozygous (516 GT) and three homozygous variant (516 TT). Figures 1a and b show the results obtained with the TaqMan assay and PCR-RFLP, respectively. Analysis of equipment and reagent cost for each method were also compared and were in the order: DNA sequencing > TaqMan allelic discrimination > PCR-RFLP. This comparison was done to assess if the different methods available for CYP2B6 G516T genotyping give the same results. Whereas the three methods have been used individually in different studies: the PCR-RFLP method

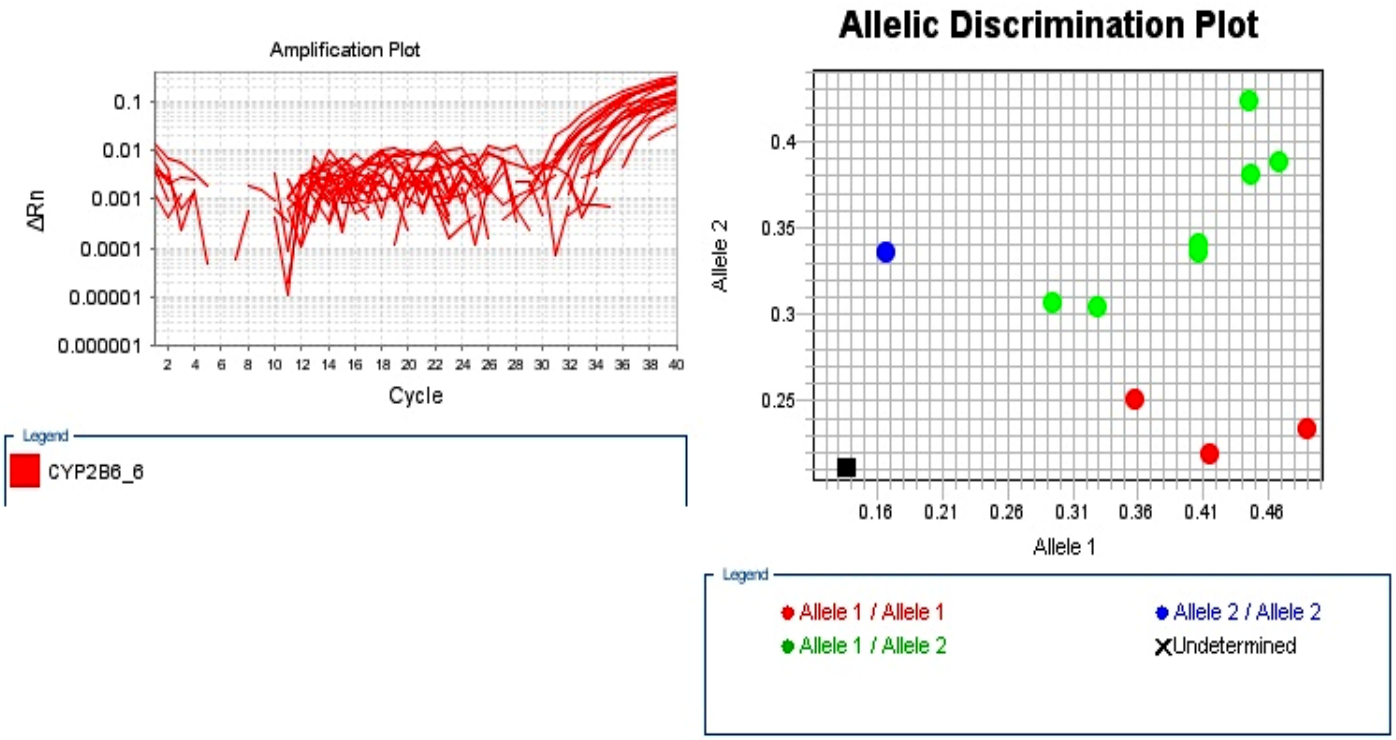

Figure 1a. Amplification plot and allelic discrimination plot for samples genotyped for CYP2B6 G516T using the TaqMan allelic discrimination assay. Each assay contains two allele-specific probes and a primer pair to detect the specific SNP target. Increase in fluorescence signal occurs when probes that have hybridized to the complementary sequence are cleaved. The fluorescence signal generated by PCR amplification indicates which alleles are present in the sample. Sequence detection software determines which alleles are present. Red = Homozygous for wild type, blue = homozygous variant, green = heterozygous. The black box shows a no DNA control.

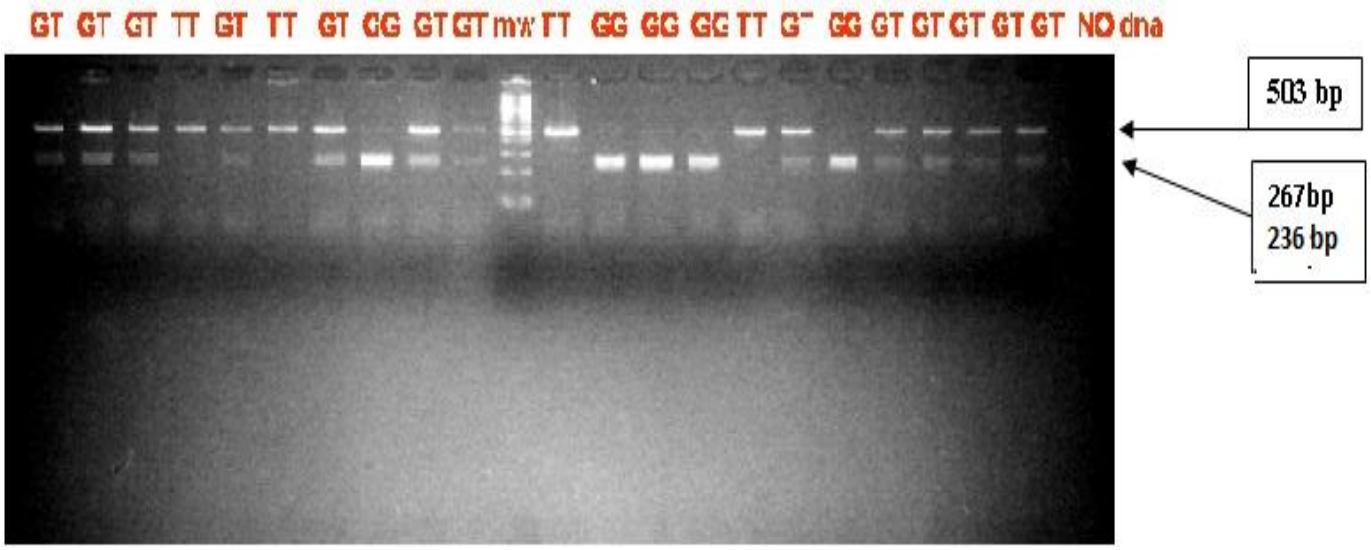

Figure 1b. PCR-RFLP based detection of CYP2B6 G516T on an ethidium bromide stained with 2% agarose gel. The restriction digestion product size is shown by the two arrows pointing to 503, 267 and 236 bp. GG = homozygous wild type (RE digestion successful no 516 G>T SNP present, 267 and 236 bp bands seen on the agarose gel); TT = homozygous mutant (516 G>T SNP present resulting in no digestion); GT, heterozygous (contains both wild type and mutant alleles, therefore all three bands are present).

(Rotger et al., 2005), the real-time PCR (RT-PCR) allelic discrimination method (Applied Biosystems, Foster City, CA) and the direct sequencing method (Mardis, 2008), this was the first study to compare the results of these

Table 1. Comparison of advantages and disadvantages of the three methods used to genotype for the CYP2B6 G516T variant and estimated input costs for each method (breakdown costs for reagents and consumables available in the supplementary material).

RFLP-PCR	TaqMan Real-Time PCR Allelic Discrimination	Direct re-sequencing
(1.) Capital equipment cost: $5 000 - $15 000	(1.) Capital Equipment Cost: $50,000-$80,000	(1) Capital Equipment Cost: $150,000-$200,000
(2.) Estimated reagents and consumables cost: $50 per sample	(2.) Estimated reagents and consumables cost: $100 per sample	(2) Estimated reagents and consumables cost: $110 per sample
(3.) Poor Precision	(3.) Increased range of detection	(3.) Simple and Robust Chemistry
(4.) Low sensitivity	(4.) No post-PCR processing	(4.) Simple Procedure, reagent components for the sequencing reaction in a ready reaction, pre-mixed format
(5.) Low resolution	(5.) Collects data in the exponential growth phase of PCR	(5.) Quantitative allele signal
(6.) Non-Automated	(6.) Increase in reporter fluorescent signal is directly proportional to the number of amplicons generated	(6.) Automated data collection and data analysis.
(7.) Size-based discrimination only	(7.) Simple and Robust Chemistry	
(8.) Ethidium bromide for staining is not very quantitative	(8.) Automated Real-Time Genotype Calling	
(9.) Post-PCR processing		

methods in the same samples. This was a necessary study to give methodological confidence to the clinical diagnostic community wishing to invest in any of these methods.

A general survey of diagnostic laboratories in Zimbabwe has shown that more than 50% have at least a PCR laboratory and a few own a real time PCR machine. This is part of an increasing trend of moving most traditional serological and enzyme linked immunosorbent assay (ELISA) based methods to DNA technology platforms. Given the potential impact of pharmacogenetics on healthcare, focus is now on the development of high-throughput methods for SNP genotyping. All current genotyping methods combine methods for allele discrimination and signal detection (Twyman, 2005). The result obtained in this study will assist most laboratories in choosing the most cost-effective platform to use, since results are reproducible with all three methods tested.

Edenburg and Liu (2009) state that an important issue in genotyping is to choose the appropriate technology for one's goals and for the stage of experiment, taking into account sample numbers and resources. With over 20% of patients in Zimbabwe requiring CYP2B6 (516G>T) genetic test guided dose adjustment (Nyakutira et al., 2008), the tremendous cost saving that can be realized from prescribing the right drug at the right dose for an efficacious and safe outcome in the use of efavirenz will have a favorable cost-benefit ratio.

In terms of capital investment and cost of reagents, the PCR-RFLP platform is the most affordable. However, this method has several drawbacks as listed in Table 1. Although, both the TaqMan allelic discrimination assay and direct re-sequencing are expensive to set-up, the simple chemistries and automated nature of these two methods make them more ideal for daily routine work, especially for a laboratory that will be doing high throughput work. Also, there is no post-PCR processing which will allow the analyst more time to work on other assays.

Adequate training is however required for the analyst to operate the sequencer and real-time PCR machine. Given the current results and despite the low investment and budgets for healthcare delivery systems in Africa, the availability of these three genotyping methods for CYP2B6 516 G>T, allow different laboratories to choose a cost effective method that best suits them. In addition, different laboratories can come together under a consortium and establish a centre where the genotyping tests can be done.

In conclusion, with the explosion of genomics, personalized medicine in part will mean checking genotypes to ensure patients get the right drug at the right dose from start of treatment (Altman et al., 2011). Therefore, it is worthwhile for laboratories in Zimbabwe and other African countries to consider investing into a genotyping method towards improvement of the health care system.

ACKNOWLEDGEMENTS

We thank Wilkins Hospital for assistance with sample collection and the Riken Institute for performing the sequencing reactions. Christopher Nyakutira's assistance with the PCR-RFLP genotyping work is also acknowledged. Funding for this work was received from the International Science Program, ISP in Sweden and EDCTP through project TA 2011.40200.052.

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