DNA Carrier Testing and Newborn Screening for Maple Syrup Urine Disease in Old Order Mennonite Communities

Stephanie M. Carleton,¹ Dawn S. Peck,² Julie Grasela,² Kristin L. Dietiker,¹ and Charlotte L. Phillips^{1,2}

Maple syrup urine disease (MSUD) is an inherited metabolic disorder caused by mutations in the branched chain α -keto acid dehydrogenase complex. Worldwide incidence of MSUD is 1:225,000 live births. However, within Old Order Mennonite communities, the incidence is 1:150 live births and results from a common tyrosine to asparagine substitution (Y438N) in the E1 α subunit of branched chain α -keto acid dehydrogenase. We developed a new DNA diagnostic assay utilizing TaqMan[®] technology and compared its efficacy, sensitivity, and duration with an existing polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) assay. Carrier testing was performed by both TaqMan technology and PCR-RFLP on DNA isolated from buccal swabs of 160 individuals as well as from buccal swabs and blood spots of nine at-risk newborns; assay time, sensitivity, and reliability were also evaluated. The TaqMan assay, like the PCR-RFLP assay, accurately determined Y438N E1 α allele status. However, the TaqMan assay appeared (1) more sensitive than the PCR-RFLP assay, requiring 10-fold less DNA (10 ng) to reliably determine genotype status and (2) faster, reducing the assay time required for diagnosis from ~12 to 5 h. TaqMan technology allowed more rapid DNA diagnoses of MSUD in the neonate, thereby reducing the likelihood of neurological impairment while enhancing health and prognosis for affected infants.

Introduction

MAPLE SYRUP URINE DISEASE (MSUD) is an autosomal recessive inborn error of branched-chain amino acid metabolism affecting the function of the branched chain α -keto acid dehydrogenase (BCKAD) complex, which processes the branched chain amino acids (BCAA) leucine, isoleucine, and valine to their α -keto acid derivatives (Danner, 1989). BCAA cannot be processed *de novo* in the human body and are, therefore, essential components of the human diet. Once in the cell, BCAA can either be incorporated into proteins or catabolized for energy using the BCKAD complex (Danner, 1989). The mammalian BCKAD contains three catalytic components. E1 contains two subunits, α and β , and functions as a decarboxylase; E2 is an acyltransferase, whereas E3 is a dehydrogenase (Chuang, 1998).

In patients with MSUD the BCAA cannot be catabolized and accumulate due to impaired oxidative carboxylation of the BCKA (Danner, 1989), leading to increased levels of BCAA and BCKA in the plasma, urine, and cerebrospinal fluid (Danner, 1989). Affected infants appear normal at birth, but as

amino acid levels rise they begin to exhibit symptoms, usually between 4 and 7 days of age (Mitsubuchi *et al.*, 2005). Symptoms include lethargy, progressive neurological damage, seizures, coma, and death if untreated. Patients left untreated also display abnormal brain histopathology and ketoacidosis (Danner, 1989), although the exact phenotype depends on the residual enzyme activity level. Rapid identification and initiation of treatment of affected infants is crucial to the prevention of neurological damage.

Although over 80 causal mutations have been identified scattered over the E1 α , E1 β , E2, and E3 genes (Nellis *et al.*, 2003), the majority of mutations are in the E1 α gene (Danner and Doering, 1998), leading to MSUD type IA with enzyme levels reduced by 70–100% (Chuang, 1998). Mutations in the E1 β , E2, and E3 genes lead to MSUD types IB (enzyme levels reduced 98–100%), type II (thiamine-responsive) and type III (combined enzyme deficiency), respectively (Chuang, 1998). The worldwide incidence of MSUD is 1:225,000 live births. However, within Missouri Old Order Mennonite communities it is 1:150 live births (Love-Gregory *et al.*, 2001). Although MSUD is genetically heterogeneous in the worldwide

¹Department of Biochemistry, University of Missouri–Columbia, Columbia, Missouri.

²Division of Medical Genetics, Department of Child Health, University of Missouri–Columbia, Columbia, Missouri.

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population, in Old Order Mennonites it results from a tyrosine to asparagine substitution (Y438N) in the *E1 α* gene due to a founder effect (Love-Gregory *et al.*, 2002). After several children were born with MSUD within a relatively short time span, the elders of the Missouri Mennonite communities requested help in identifying families at risk of having a child with MSUD and with early diagnosis of MSUD in affected infants in an attempt to reduce morbidity (Love-Gregory *et al.*, 2001). In response, our laboratory designed a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) assay (Love-Gregory *et al.*, 2001), which was culturally permissive in accordance with strict religious restrictions against prenatal diagnosis and had advantages over traditional amino acid level tests. The assay allowed identification of couples at risk of having a child with MSUD before pregnancy as well as perinatal testing of at-risk infants. In addition, Old Order Mennonites do not carry health insurance, so it was imperative the assay be fairly inexpensive so as not to be economically restrictive. Finally, it was critical that the assay be relatively fast, as the goal was to diagnose MSUD in affected infants before the onset of clinical disease. Although the PCR-RFLP assay proved successful at identifying Y438N allele status in 570 individuals, including 12 at-risk newborns, this methodology also had several limitations. Specifically, it is labor intensive, the results can be difficult to obtain with relatively low amounts of DNA, and the 12–14 h required to complete the assay can be problematic. This time restraint is further compounded by cultural restrictions of the Mennonite community as many Mennonites live in rural areas several hours from major medical centers and often do not drive.

TaqMan[®] technology (Applied Biosystems, Foster City, CA) presents a unique opportunity to improve assay sensitivity and reduce the time required to identify affected infants. TaqMan assays are PCR based and incorporate laser scanning technology to excite a fluorescent dye attached to detection probes with complementary sequences to both normal and variant forms of an allele (Livak, 1999). Each of the two probes has a unique fluorescent reporter dye attached to the 5' end and a quencher attached to the 3' end (Livak, 1999). When the probe is intact, the quencher prohibits emission of the reporter dye. However, during PCR extension the DNA polymerase cleaves the reporter dye from the probe, thereby permitting the reporter dye to emit its specific fluorescence (Livak, 1999). By measuring the relative amounts of the two dyes, allele status can be determined. The goal of the project presented here was to compare the PCR-RFLP and TaqMan assays in 160 individuals, including nine at-risk newborns, in an attempt to increase assay sensitivity and reduce the time required to determine Y438N allele status.

Materials and Methods

Sample collection

Volunteers were recruited from Old Order Mennonite communities throughout Missouri and Illinois by the Division of Medical Genetics Metabolic Clinics at the University of Missouri School of Medicine. Clinics servicing these communities were informed of the study by mail and/or phone and asked to offer testing for the Y438N allele to their Mennonite patients. As these tend to be small communities, many patients were recruited by word of mouth within the com-

munities. All willing participants were included regardless of age, sex, or marital status. Each study participant was provided information about the study in accordance with an approved Health Sciences Institutional Research Review protocol, and informed consent obtained before collection of two self-obtained buccal swabs containing cheek epithelial cells. For newborn testing, parental informed consent was obtained and midwives collected two buccal swabs as well as blood from the heel and/or umbilical cord of the newborn. The samples were then transported at room temperature in sealed containers to our laboratory by a volunteer driver for processing.

DNA isolation

Sterile 1× phosphate-buffered saline was added to each buccal swab and vortexed every 10 min for a total of 60 min. Cells were then pelleted and lysed before adding 300 μ L of Instagene (Bio-Rad, Hercules, CA) (Love-Gregory *et al.*, 2001).

PCR-RFLP assay

Mismatch PCR amplification utilized primers (5' sense primer: 5' CCACCTGCAGACCTACGGGAGTAC 3'; 3' antisense primer: 5' GCCCTGGCCGCTGAGTACTTTAGAGG 3') designed to identify the Y438N mutation via loss of the *ScaI* restriction site (5'AGTACT3') and to include an internal control *ScaI* restriction site as previously described (Love-Gregory *et al.*, 2001).

TaqMan assay

Isolated DNA was also subjected to TaqMan analysis using PCR primers (5' sense primer: 5' CCGCCACCTGCAGACCT 3'; 3' antisense primer: 5' GCTGAGCAGGTCTCACTTATCGA 3') designed to amplify the region containing the Y438N allele and sequence-specific fluorescent probes (Wt: 5' 6FAM-AGCACTACCCACTGGA 3'; MSUD: 5' VIC-AGCAC AACCCACTGG 3') designed to bind only to PCR fragments of the complimentary sequence. Amplification was done in a 50 μ L volume according to manufacturer's instructions on an ABI Prism 7000 (Applied Biosystems) for 2 min at 50°C, 10 min at 95°C, and 40 cycles at 95°C for 15 s and at 60°C for 1 min. Genotypes were determined using probe-specific fluorescence (Fig. 1).

Results

To date, 151 individuals have volunteered for carrier testing for the Y438N allele using both PCR-RFLP and TaqMan assays. Additionally, nine at-risk newborns have undergone perinatal testing using both methodologies. The TaqMan assay, like the PCR-RFLP assay, accurately detected the presence or absence of the Y438N allele (Fig. 1). However, TaqMan required less DNA (10 ng) to reproducibly determine allele status than did the PCR-RFLP assay (100 ng; Table 1). TaqMan was also more reliable, requiring retesting of only 9% of samples (15/160) to verify Y438N allele status as compared to 46% (74/160) of samples for the PCR-RFLP assay (Table 1). The TaqMan assay is also faster than the PCR-RFLP assay. Both assays require DNA isolation from buccal swabs and PCR, totaling four to five hours. However, the PCR-RFLP assay requires visualization of PCR products by agarose gel electrophoresis (1 h), restriction endonuclease digestion with

FIG. 1. Representative images of polymerase chain reaction–restriction fragment length polymorphism (A) and TaqMan (B) assays. (A) 4% agarose gel demonstrating the presence of a 170 bp band (Y438N allele) and/or a 147 bp band (normal allele). The presence of both bands indicates a carrier (Y438N/+). “NTC” indicates a no DNA control, and “uncut” indicates the 186 bp polymerase chain reaction product before *ScaI* restriction endonuclease digestion. (B) Normal:Y438N ratios were used to determine allele status. Normal or Y438N alleles had ratios of at least 2:1, whereas carriers had allele ratios of ~1:1. Standards were harvested from commercially available cell lines (ATCC; Normal Standard: GM00651; Y438N/+ Standard: GM00650; Y438N Standard: GM01099).

ScaI (3 h), and separation of restriction endonuclease products by agarose gel electrophoresis (2 h; Fig. 1A). These additional steps add six hours to the time required to confirm Y438N allele status.

Discussion

Our results demonstrate that both the PCR-RFLP and TaqMan assays can accurately determine Y438N allele status within 24 h of birth. However, the TaqMan assay is 10-fold more sensitive than the PCR-RFLP assay, requiring only 10 ng of DNA to reliably determine genotype status. As most buccal swabs, particularly those for carrier detection, are self-swabs collected in the home without direct supervision by trained personnel, the amount of DNA collected varies greatly from person to person. Therefore, it is imperative the assay be sensitive enough to determine allele status from relatively

TABLE 1. COMPARISON OF POLYMERASE CHAIN REACTION–RESTRICTION FRAGMENT LENGTH POLYMORPHISM AND TAQMAN ASSAYS IN 160 INDIVIDUALS

	PCR-RFLP	TaqMan®
Samples requiring repeat testing ^a	74	15
% Repeat samples	46%	9%
Assay sensitivity	100 ng DNA	10 ng DNA
Time required to complete assay	11–14 h	4–5 h

^aIncludes nine at-risk newborns.

^aSome samples required multiple repeats.

PCR-RFLP, polymerase chain reaction–restriction fragment length polymorphism.

small amounts of DNA. The TaqMan assay was also more reliable, requiring fewer retests to determine Y438N allele status, thus reducing the cost and time required to perform the assay. The PCR-RFLP assay often has to be repeated due to insufficient DNA concentration (17/160 samples), gel electrophoresis visualization issues (7/160 samples), or contamination/miscellaneous issues (50/160 samples). As the TaqMan assay has fewer steps, the likelihood of an error or technical problem is reduced. On the rare instance that the TaqMan assay had to be repeated (15/160 samples), reduced fluorescence due to probe age was found to be the culprit. The TaqMan assay is also much faster than the PCR-RFLP assay, reducing diagnostic time from 12 to 4–5 h. As prenatal testing is not an option in this population and the majority of Missouri Old Order Mennonites do not drive yet live in communities removed from major medical centers, it is often several hours after the birth before samples arrive for testing. As our goal is to diagnose MSUD in an affected infant within 24 h of birth, time is of the essence as the earlier an affected infant is put on a protein-restricted diet, the lower the blood levels of ketoacids and the better the prognosis (Edelmann *et al.*, 2001; Mitsubuchi *et al.*, 2005; Simon *et al.*, 2006; Packman *et al.*, 2007). Additionally, there are more than 7,000 real-time PCR machines in circulation in North America capable of performing the TaqMan assay, with about 300 of those in clinical settings (Applied Biosystems; personal communication). Therefore, this assay has the potential to be made easily available to Mennonite communities throughout the country.

In conclusion, an effective and culturally permissive DNA test is available for the Y438N E1 α gene defect responsible for MSUD within the Old Order Mennonite population using two distinct methodologies. Both assays enable identification of families at risk for MSUD as well as the diagnosis of MSUD in affected asymptomatic infants. However, the TaqMan assay is more sensitive, is less expensive, and allows for faster diagnosis than the traditional PCR-RFLP assay, thus reducing the likelihood of neurological impairment while providing more time for affected infants to seek medical care before the onset of clinical disease.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Charlotte L. Phillips, Ph.D.

Departments of Biochemistry and Child Health

University of Missouri–Columbia

117 Schweitzer Hall

Columbia, MO 65211

E-mail: phillipscl@missouri.edu