Brazilian Journal of Medical and Biological Research (2004) 37: 295-299
ISSN 0100-879X Short Communication

Newborn screening for biotinidase deficiency in Brazil: biochemical and molecular characterizations

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Abstract

Biotinidase deficiency is an inherited metabolic disorder characterized by neurological and cutaneous symptoms. Fortunately, it can be treated and the symptoms prevented by oral administration of the vitamin biotin. Using dried blood-soaked filter paper cards, biotinidase activity was determined in the sera of 225,136 newborns in Brazil. Mutation analysis performed on DNA from 21 babies with low serum biotinidase activity confirmed that 3 had profound biotinidase deficiency (less than 10% of mean normal sera biotinidase activity), 10 had partial biotinidase deficiency (10 to 30% of mean normal serum activity), 1 was homozygous for partial biotinidase deficiency, 4 were heterozygous for either profound or partial deficiency, and 3 were normal. Variability in serum enzyme activities and discrepancies with mutation analyses were probably due to inappropriate handling and storage of samples sent to the laboratory. Obtaining an appropriate control serum at the same time as that of the suspected child will undoubtedly decrease the false-positive rate (0.09%). Mutation analysis can be used to confirm the genotype of these children. The estimated incidence of biotinidase deficiency in Brazil is about 1 in 9,000, higher than in most other countries. Screening and treatment of biotinidase deficiency are effective and warranted. These results strongly suggest that biotinidase deficiency should be included in the newborn mass screening program of Brazil.

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Research supported in part by the Safra Research Fund (B.W.) at Connecticut Children's Medical Center and by a General Clinical Research Center Grant to the University of Connecticut Health Center (MO1RR06192) from the National Institutes of Health.

Received March 11, 2003
Accepted October 24, 2003

Key words
- Newborn screening
- Biotinidase deficiency
- Biotinidase
- Mutations
- Enzyme assay

Biotinidase deficiency is an autosomal recessive inherited disorder of the recycling of biotin, an essential water-soluble vitamin. Biotin is the coenzyme of four carboxylases involved in amino acid catabolism, fatty acid synthesis, and gluconeogenesis (1). Deficient activity of this enzyme is the primary defect in most children with biotin-responsive, late-onset multiple carboxylase deficiency (2). Symptoms of the disorder usually occur at several months of age and are characterized by neurological and cutaneous abnormalities, including hypotonia, lethargy, seizures, ataxia, developmental delay, hearing loss and

vision problems, skin rash and alopecia (3). These children can develop metabolic ketoacidosis, hyperammonemia, and organic aciduria (3,4). These features are caused by secondary deficiencies of the activities of the biotin-dependent enzymes, propionyl-CoA carboxylase, pyruvate-CoA carboxylase, and ß-methylcrotonyl-CoA carboxylase (1). If not treated with biotin, children with the enzyme deficiency may become comatose and die. Symptoms can be prevented if biotin therapy is initiated early (1). Because biotinidase deficiency meets the criteria for inclusion in newborn screening programs, at least 25 countries have included the disorder in their programs (5). Brazil is a country with multiple ethnic groups and the incidence of biotinidase deficiency is unknown. We now report our experience with screening newborns for biotinidase deficiency from October 1995 to November 1999 in a private neonatal screening program in Southern Brazil.

Some private programs in Brazil offer a great number of tests for genetic and infectious diseases. Based on the high incidence of biotinidase deficiency found through the currently available private screening programs in Brazil, more widespread screening will undoubtedly identify many more children with the disorder. Compared to other disorders that are currently screened country-wide through public supported programs (phenylketonuria, congenital hypothyroidism and hemoglobinopathies), screening for biotinidase deficiency would be cost-effective.

Blood-soaked filter paper cards (filter paper SS 903 from Schleicher and Schuell, Keene, NH, USA) were obtained from 225,136 babies between the ages of 2 and 30 days (median age: 13 days) from throughout Brazil. To identify potentially biotinidase-deficient children, biotinidase activity was determined in punched disks from the cards using a qualitative colorimetric assay in which biotinyl-p-aminobenzoate is the substrate (6). Confirmation of biotinidase activity in serum was obtained for babies suspected of having the deficiency and for their parents by a quantitative colorimetric assay using biotinyl-p-aminobenzoate as described previously (2). Normal serum enzyme activity ranged from 4 to 10 µmol p-aminobenzoate formed ml^{-1} min^{-1}. Blood for DNA was obtained from 21 children with serum enzyme activity below 30% of the mean activity of normal children to confirm and identify mutations by direct sequencing (7,8). Mutation analyses were performed by direct sequencing of the coding regions of the biotinidase gene including exon/intron boundaries. Novel mutations were confirmed by sequencing the entire gene and by identifying the alterations in the DNA of the parents when available.

Of the 225,136 babies screened, 272 had absent or low biotinidase activity in the initial blood spot screening. All families were contacted and serum for quantitative serum enzyme activity was requested. Samples were received from 240 babies. Requested serum samples were supposed to be immediately frozen and shipped to the laboratory on dry ice. However, because of the size of Brazil and the lack of facilities for shipping frozen samples, most of them were thawed when received by the laboratory two to five days after blood was obtained. Biotinidase activity was measured as soon as the samples reached our laboratory. Normal activities were found in 204 babies and 36 children had less than 30% of mean normal serum activity in their initial serum test; 14 had enzyme activity below 10% and 22 had enzyme activity between 10 and 30%. Most of these children had multiple serum enzyme activities determined during the first two years of life. Whole blood was requested from these 36 children and their parents for mutation analysis. Only 21 (58%) of the families agreed to have mutation analysis. The results of the biochemical and molecular studies of these 21 children and their

parents are presented in Table 1. None of the families were consanguineous. Clinical follow-up was performed by telephone with the physicians and parents of the children. All children on biotin therapy have remained asymptomatic.

As can be seen in Table 1, there were discrepancies in the serum enzyme activities of some of the children. Because there are multiple factors that can result in falsely low enzyme activity, the highest enzyme activity of a child was likely to represent the true activity. Based on these enzyme results, it would be expected that some of the children initially thought to have profound or partial biotinidase deficiency would be heterozygous for the disorder or would not have a mutation at all. Mutational analyses confirmed or excluded the diagnosis of biotinidase deficiency in all children tested. Based on these mutation studies, of the 21 children tested, 3 were confirmed to have profound biotinidase deficiency, 10 were confirmed to have partial biotinidase deficiency, 1 was found to be homozygous for partial biotinidase deficiency, 4 were heterozygous for either profound or partial deficiency, and 3 were found to be normal. Only the 14 children confirmed to have profound or partial biotinidase deficiency required continued biotin treatment.

We identified four novel mutations in our population (1314T>A, 133G>A, 595G>A and 236G>A). All of these mutations appear to cause profound biotinidase deficiency based on serum enzyme activity. The 1314T>A

Table 1. Biochemical and molecular characterization of children suspected of having biotinidase deficiency by newborn screening.

Patient	Mutation 1	Enzyme alteration 1	Mutation 2	Enzyme alteration 2	Classification	Patient's biotinidase activity[a]	Mother's biotinidase activity	Father's biotinidase activity
P724	98-104d7i3	Frameshift	235C>T	79R>C	Profound deficiency	0,0,0	2.9	3.9
P739	755A>G	252D>G	755A>G	252D>G	Profound deficiency	0,0,0.2	3.7	7.3
P835	**595G>A**	**199V>M**	**1314T>A**	**438Y>stop**[b]	Profound deficiency	0.7,0.8	5.0	5.4
P815	98-104d7i3	Frameshift	1330G>C	444D>H	Partial deficiency	1.6,3.2,1.0	8.5	NA
P818	98-104d7i3	Frameshift	1330G>C	444D>H	Partial deficiency	1.6,2.0,1.8	5.0	5.3
P850	98-104d7i3	Frameshift	1330G>C	444D>H	Partial deficiency	1.7,1.8	4.3	4.9
P730	100G>A	Splice site	1330G>C	444D>H	Partial deficiency	1.5,1.5,1.7,0.6	5.6	3.8
P715	184G>A	62V>M	1330G>C	444D>H	Partial deficiency	2.2,0.8,1.6	6.0	1.6
P853	**595G>A**	**199V>M**	1330G>C	444D>H	Partial deficiency	2.1	4.5	4.4
P736	755A>G	252D>G	1330G>C	444D>H	Partial deficiency	0,1.5,1.5,2.2	9.4	6.2
P718	511G>A:1330G>C	171A>T;444D>H	1330G>C	444D>H	Partial deficiency	0.1,0.7,1.9	3.8	5.6
P721	511G>A:1330G>C	171A>T;444D>H	1330G>C	444D>H	Partial deficiency	0.9,1.9	6.0	7.0
P733	511G>A:1330G>C	171A>T;444D>H	1330G>C	444D>H	Partial deficiency	0.9,0,1.8	4.8	6.2
P856	1330G>C	444D>H	1330G>C	444D>H	Homozygous for partial deficiency	2.5	4.5	5.5
P811	133G>A	45G>R	None		Heterozygous for profound deficiency	3.2,0.9,0.1	6.7	5.3
P824	**236G>A**	**79R>H**	None		Heterozygous for profound deficiency	0,4.2	9.4	4.5
P821	1330G>C	444D>H	None		Heterozygous for partial deficiency	1.4,5.1,5.2	5.2	8.9
P859	1330G>C	444D>H	None		Heterozygous for partial deficiency	1.3,4.8	6.6	6.2
P727	None		None		Normal	0,8.5	7.2	8.4
P829	None		None		Normal	0.3,7.2	7.6	6.9
P832	None		None		Normal	0.7,5.8	6.6	10.2

[a]Multiple serum enzyme determinations. Units of activity are reported as nmol min^{-1} ml $serum^{-1}$. Normal activity is 4.4 to 12. [b]Also has 1413T>C polymorphism. Mutations in bold are novel mutations. NA = not available.

mutation results in an alteration of a tyrosine residue to a termination codon in the latter portion of the enzyme protein. The 133G>A mutation is a subtle mutation resulting in an alteration of a glycine to alanine. This mutation has been observed in an allelic double mutation previously, but not alone (9). The 595G>A mutation was observed in two children and results in a change from a valine to methionine. This mutation resides in a region where multiple other missense mutations have been found. The 236G>A mutation results in a change of an arginine to a histidine and is located in a region of biotinidase that is homologous with the active site of bacterial aliphatic hydrolases (10).

Biotinidase deficiency is diagnosed by demonstrating deficient enzyme activity in serum. Children with profound biotinidase deficiency have serum enzyme activity less than 10% of mean normal activity (1). These children are most likely to become symptomatic if they are not treated with biotin. Children with partial biotinidase deficiency have 10 to 30% of mean normal serum biotinidase activity. These children may develop symptoms if they are stressed by an infection (11). Both groups of children are now routinely treated with biotin. In our study of 21 children suspected of having either profound or partial biotinidase deficiency, 14 or 66.7% were confirmed to have the disorder based on mutation analysis. The other children were shown to be carriers of mutations causing either profound or partial deficiency or did not have any mutations at all. In addition, the screening revealed four novel mutations, three missense mutations, and one that resulted in premature termination.

The incidence of profound and partial biotinidase deficiency worldwide is estimated at about 1 in 60,000 (5). If we consider 22 cases in the present adjusted sample size of 198,694 newborns, then the incidence of biotinidase deficiency in Brazil is about 1 in 9,000 births. Either estimation indicates that the incidence of biotinidase deficiency in Brazil is one of the highest known.

Our recall rate for second screening samples of 0.12% is considerably higher than reported by most other laboratories (5,12). This problem is usually attributable to poor impregnation of blood on filter paper, increased humidity, failure to allow the filter paper to dry sufficiently before packing in plastic bags, or heat exposure. The problem with discrepancies in quantitative enzyme determinations is usually failure to rapidly freeze and ship the samples on dry ice or failure to store the samples in the laboratory at -80°C if not immediately assayed. As stated above, samples were not usually frozen when received by the laboratory. Unfortunately, most samples were shipped at room temperature and were two to five days old when they arrived. Routinely, samples were assayed immediately after they were received. The inadequate handling and shipping of the samples are undoubtedly the cause for the high false-positive rate and the disparity in reproducibility in serum enzyme activities. These factors also explain the discordance between the low serum biotinidase activities and the failure to find mutations in some of the children who initially had low enzyme activities.

Clearly, when multiple serum enzyme activity determinations are performed, the highest activity is most representative of the child's true genotype. One remedy for this problem is to request a control serum sample from an unrelated individual, presumed to have normal activity, obtained at the same time as the sample from the suspected enzyme-deficient child. In addition, samples should also be obtained from the parents to confirm heterozygosity. If the control activity is low, then the activities in the sera from the child and parents should be suspected. If the control activity is well within the normal range, the activity sample from the child and parents may be interpretable. Final confirma-

tion of biotinidase deficiency can be obtained using mutation analysis, but this is an expensive, inconvenient and time consuming procedure.

Parental testing was not helpful in discerning which children were likely to have biotinidase deficiency as it usually is when samples are frozen on arrival to the laboratory. Although only 1 of the 6 parents of children who was found to be normal had serum biotinidase activity in the heterozygous range, 3 of 6 parents of children confirmed to have profound biotinidase deficiency had normal enzyme activities. Perhaps the parental enzyme activities would be helpful in deducing the genotype if a simultaneously obtained normal control was included for comparison.

Despite the high number of samples with false-positive results in filter paper when compared with the true positive cases, newborn screening for biotinidase deficiency appears to be reasonable and effective. Alterations in obtaining appropriate controls should help to eliminate or minimize discrepancies and variability in determining true deficiencies. Final confirmation can be achieved by mutation analysis that does not depend on freezing the samples on shipment. The preliminary estimated incidence of biotinidase deficiency in Brazil is relatively high compared to most other countries and warrants inclusion of biotinidase deficiency in the group of disorders screened for in Brazil.

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