Perspectives for Early Genetic Screening of Lactose Intolerance: -13910C/T Polymorphism Tracking in the *MCM6* Gene

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Abstract: *Introduction*: For many years Lactose intolerance has been, considered as a universal problem in many children and adults. *Objective*: The aim is to investigate the prevalence of polymorphism -13910C/T, in a neonatal tracking, for early diagnosis of lactose tolerance/intolerance. *Materials and Methods*: In a cross-sectional study of 310 Brazilian newborns, DNA was extracted from leukocyte umbilical cord and specific primers were used to amplify the region that encloses the -13910C/T polymorphism of the *MCM6* gene, using the polymerase chain reaction and the restriction fragment length polymorphism tests. *Results*: One hundred and sixty (52%) male newborns and 150 (48%) female new borns were evaluated. Out of these, 191 (62%) presented CC genotype (lactose intolerant), 95 (31%) CT genotype, and 24 (7%) TT genotype, comprising a total of 119 (38%) lactose tolerant newborns. Accordingly the newborns´ gender distribution in relation to the phenotypes has been found; 97 (32%) of male gender and 94 (30%) of female gender lactose intolerant, and 63 (20%) male and 56 (18%) female lactose tolerant newborns, not being such distribution statistically significant (p = 0.801). *Conclusions*: The molecular analysis made possible the identification of the presence or absence of lactase persistence variant in the Brazilian newborns. The neonatal molecular diagnosis can optimize the follow-up of positive results in newborn screening for lactose intolerance.

Keywords: Molecular analysis, *MCM6* gene, lactase, lactose malabsorption, hypolactasy.

INTRODUCTION

Approximately 70% of the world population has lactase primary deficiency. This percentage varies according to ethnicity and is related to the use of dairy products in the diet, resulting in the genetic selection of individuals with the ability to digest lactose. In populations with the predominance of dairy products in their diet due to the pastoral tradition, particularly, in the North of Europe, only 2% of the population has primary lactase deficiency. Contrarily, according to the anthropologic studies, people with high prevalence of adult lactose malabsorption are those, who have agricultural and hunting tradition and who never drank milk or who started taking it only a few thousand year ago, but as fermented dairy products and, therefore, poor in lactose [1-4].

Primary deficiency prevalence is from 50% to 80% in the Hispanic population, 60% to 80% in Negroids, mainly Africans from Hamite origin, and Ashkenazi Jewish people and almost 100% in the Americans and Asian Indians, besides the Eskimos. The groups with intermediate prevalence are cross-breed from the groups mentioned: African cross-breed between Bantu and Hamite, cross-breed from the Europeans with Orientals and with Indians. The beginning age and the prevalence differ among many populations. Approximately 20% of Hispanic, Asians and Black children below five years of age have the evidence of lack of lactase and lactose malabsorption while white children, typically, do not develop lactose intolerance symptoms up to the age of four to five years [1-4].

Despite many studies have been carried out worldwide [1,3-7], there are few data published pertaining the lactose intolerance prevalence in the Brazilian population. According to these data, there has been a frequency of approximately 50% in the Caucasian, 85% in the Negroids, and 100% in the Orientals, being these results, in accordance with the ethnic race of the Brazilians and according to the studied region [8-12].

Adult lactose malabsorption is hereditarily inherited by autosomal recessive gene. The opposite condition - the persistence of lactase activity in adult life - is inherited by autosomal dominant gene and it is probably a polymorphism in a regulatory gene, which results in permanent capacity of digesting sugar from milk all lifelong. The persistence of lactase activity represents a genetic polymorphism in the human population that involves the regulation of development [13,14].

Several polymorphisms have been found in introns and exons of the lactase gene and in its promoter region, but none of them, consistently correlates with the persistence/ non-persistence of lactase [15]. A recent finding has been described by a group from Finland, in which two polymorphisms have been found in the introns 9 and 13 of the *minichromosome maintenance 6* gene (*MCM6*, OMIM – 601806) [13]. The polymorphisms, numerated from the beginning codon – ATG – from *LCT* gene, are the -13910C/ T in intron 13, located 14 Kb upstream of the lactase gene

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and the polymorphism -22018G/A in intron 9, located 22 Kb upstream of the gene, which correlate 100% and 97% with the lactose tolerance/intolerance phenotype [13, 16, 17] respectively. The homozygote genotypes CC and GG have low lactase levels (non-persistent to lactase) therefore being intolerant to lactose. The homozygote genotypes TT and AA have high levels of lactase (lactase persistent) and the heterozygotes CT and GA intermediate lactase levels, being all of them tolerant to lactose. Therefore, the T allele is present in all individuals with lactase persistence (tolerant to lactose) and absent in those with non-persistence (lactose intolerant) [18].

The *MCM6* gene has been scanned by fluorescence *in situ* hybridization in chromosome 2, in position 2q21 [19], being expressed in a variety of adult and fetal human tissues, having an important role in the regulation of the DNA replication. The lactase persistence/non-persistence is, therefore, associated with a non-coding variation in the *MCM6* gene situated upstream of the lactase gene, in a region that appears to act as a *cis* element capable of enhancing differential transcriptional activation of the lactase promoter region [14].

Although DNA analyses, specific in allowing T/C and G/A polymorphisms identification, have made possible the elucidation of the molecular base involved in the regulation of lactase promoting transcription (LCT), with consequent importance in the diagnosis of persistence/non-persistence of lactase activity, there are few studies in developing countries, especially in Brazil [20-22] and, up to now, none on newborns.

This study aimed at investigating the prevalence of -13910C/T polymorphism in a neonatal screening, for an early diagnose of lactase tolerance/intolerance in order to provide adequate specific therapeutic measures.

MATERIALS AND METHODS

During the period from July to October 2009 a cross-sectional study was carried out, in which, 310 both male and female newborns were evaluated by molecular tests, after parents signing the clarified free consent term, approved by the Research Ethics Committee of FAMERP.

The newborns selected for the study obeyed the following inclusion criteria: a) born during the study period, either by vaginal birth or surgical delivery, b) Apgar index ≥ 7 in the 1st minute, c) without apparent congenital malformation, and d) born alive with gestational age equal or superior to 38 weeks.

A total of 4.0mL of blood was collected from the umbilical cord, after its tying in a Vacutainer® tube containing anticoagulant (EDTA). The genomic DNA was extracted from the newborns´ blood samples by using the GE Illustra Blood Genomicprep Mini Spin Kit™ (GE Healthcare UK Limited), according to the manufacture´s protocol.

In order to detect the -13910C/T polymorphism, the genomic DNA fragment, which comprehends the polymorphism region in intron 13 of *MCM6* gene, was amplified by the PCR test, in the Bioer Technology® (TC-XPG model) thermociclator. The following sequence of primers was used (access OMIM-#AY220757): forward primer *MCM6-LCT* (F) - AAG ACG TAA GTT ACC ATT TAA TAC (26533 to 26556 position) and reverse primer *MCM6-LCT* (R) - CGT TAA TAC CCA CTG ACC TAT CCT (26748 to 26725 position), and the conditions for the PCR reaction were according to those described in the literature [21], with changes in the annealing temperature, in the cycles number of PCR and in the duration of cycles. Genomic DNA (200 ng) was used as template in a reaction of final volume of 25 µL, containing 10 pmol of each primer and all other reagents from *FideliTaq™ PCR MasterMix (2X) (GE Healthcare®)*, according to manufacturer's protocol. The amplification was performed as follows: initial denaturation at 94ºC for 3 min followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 60ºC for 1 min and extension at 72ºC for 2 min. The final extension at 72ºC was for 10 min.

As a product for the PCR reaction, a fragment of 216 bp was amplified, which was, afterwards, submitted to the restriction analysis by the RFLP test, using the BsmFI [21] enzyme (New England Biolabs)®, at 65°C, for 2h30min. When the polymorphism is present in both alleles (homozygote sample), the PCR product is digested by the BsmFI enzyme, due to the recognition of the enzymatic restriction site, in fragments of 126 bp and 90 bp and, when the polymorphism is present in only one allele (heterozygote sample), the product of the PCR of the mutant allele is digested in two fragments of 126 bp and 90 bp and of the normal allele is not digested, presenting a fragment of 216 bp. In the polymorphism absence, in both alleles, the PCR product is not digested, presenting only the 216 bp fragment, because there is no recognition of the enzyme restriction site, due to the non substitution of the nitrogenate bases C/T, in the position -13910 of the *MCM6* gene.

The products from both reactions, PCR and RFLP, were analyzed by agarose gel electrophoresis 2% in TBE 1x buffer, containing ethidium bromide, in 0.5µg/mL concentration, submitted to ultraviolet lighting, to assure the success of the reaction and the gel was photodocumented.

Statistical Analysis

Percentages were calculated, being the results expressed in (%) and using the Chi-Square Test for comparison among the variables.

Ethical Aspects

According to the Regulating Rules of Research on Human Beings, Resolution 196/96 from the Health Ministry, the present study was approved by the Research Ethics Committee of the Medical School of São José do Rio Preto, SP (CEP-FAMERP), under Resolution #061/2009.

RESULTS

According to the inclusion and exclusion criteria, 310 newborns were selected, being 160 (52%) male and 150 (48%) female. After the enzymatic digestion for the -13910C/T polymorphism identification, the obtained results expressed the following genotypes: a) CC: related to the phenotype of non-persistence of the lactase enzyme activity - lactose intolerance, b) CT and TT: related to the phenotype of persistence of the lactase enzyme activity - lactose

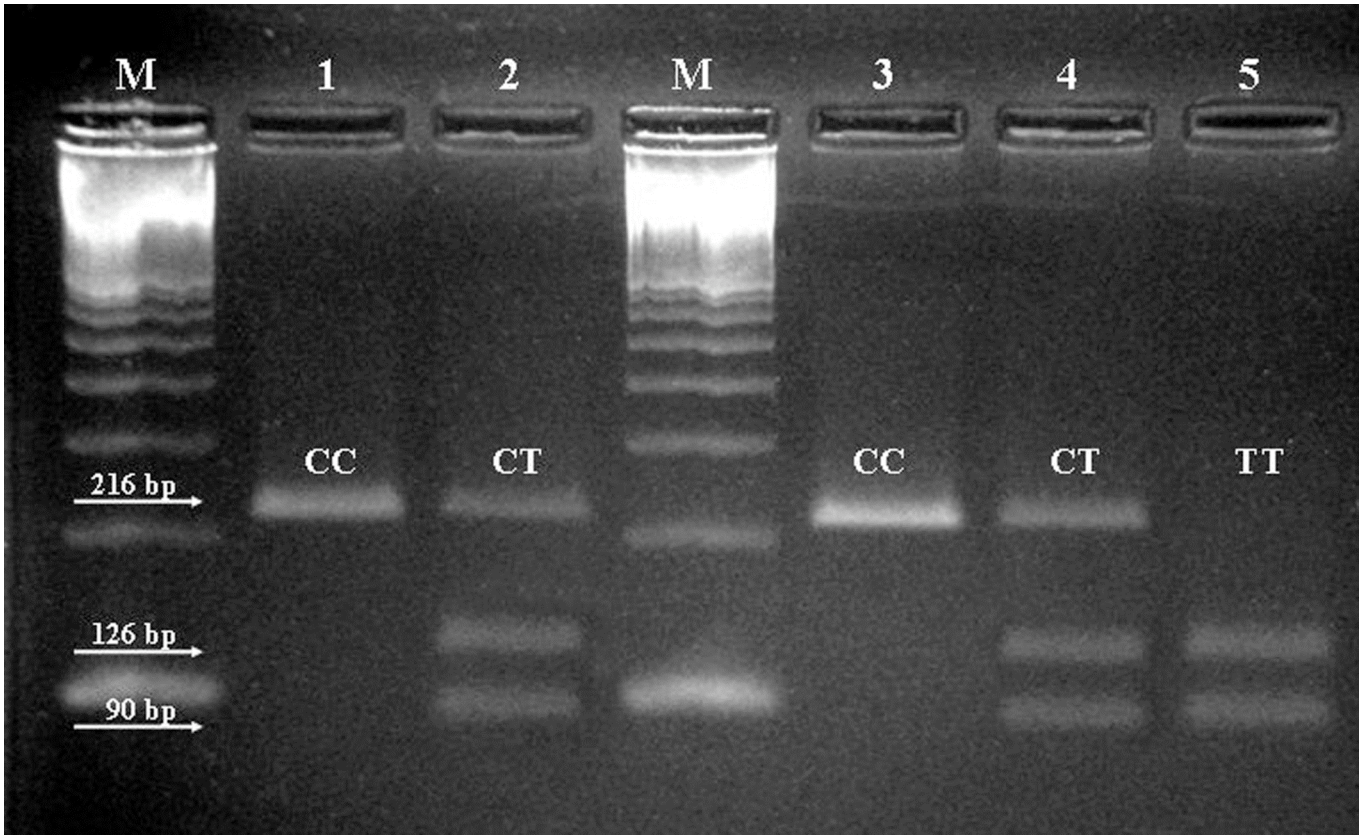

Fig. (1). RFLP products of 216 bp, 126 bp and 90 bp of *MCM6* gene, analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide. Column 1: positive control (CC - lactase non-persistence). Column 2: negative control (CT - lactase persistence). Columns 3 to 5 - samples of the study: Column 3: CC (lactase non-persistence); Column 4: CT (lactase persistence); Column 5: TT (lactase persistence). M-100 bp DNA ladder.

tolerance (Fig. **1**). Thus, 191 newborns (62%) were identified with CC genotype, 95 (31%) with CT genotype and 24 (7%) with TT genotype, comprising a total of 119 (38%) newborns with lactose tolerance. The allelic frequency C and T were, respectively, 77% and 21% of the analyzed alleles.

The distribution of newborns gender in relation to the phenotypes was performed, finding 97 (32%) of the male gender and 94 (30%) of the female gender lactose intolerant, and 63 (20%) of male newborns and 56 (18%) of female ones lactose tolerant, being, this distribution, not statistically significant (p = 0.801).

These results are shown in Tables **1** and **2**.

Table 1. Distribution of the Number of Newborns (n = 310) in Relation to Genotype and Genotypic Frequency in Percentages

Genotype	Newborns (n)	Genotypic frequency (%)
CC	191	62
CT	95	31
TT	24	07
Total	310	100

Table 2. Distribution of the Gender of Newborn (n = 310) in Relation to Phenotype in Percentages

Phenotype	Gender		Total (%)	*p**
	Male (%)	Female (%)		
CC- lactose intolerant	97 (32)	94 (30)	191 (62)	0.801
CT and TT - lactose tolerant	63 (20)	56 (18)	119 (38)	
Total	160 (52)	150 (48)	310 (100)	

*Chi-Square Test.

DISCUSSION

For many years, lactose intolerance has been, considered as a worldwide problem in many children and adults. Although it rarely poses a threat to life, the symptoms of lactose intolerance can lead to a significant discomfort, worsening of life quality, school difficulties, interruption of entertaining or sports activities, being absent from work, all of these with an individual, family and social cost [2].

The ingestion of lactose in certain susceptible individuals can cause varied abdominal symptoms, which can be treated with milky restriction or enzymatic reposition, depending on

the quantity of lactose consumed and/or deficiency degree of lactase. Children under suspicion of lactose intolerance can be clinically diagnosed only by eliminating lactose from their diet or by complementary exams which include the expired hydrogen test and/or intestinal invasive biopsy to determine lactase concentration (and other disaccharides, when necessary), which are very often refused by parents. Nowadays, genetic tests are being performed to investigate lactose intolerance, not only for being considered non-invasive but also by the high sensitivity and specificity for molecular study, as performed in the present work [13,18,22-25]. This study is a brief description of the first neonatal screening on lactose intolerance performed in Brazil.

A prevalence of 62% (1:1.62) of newborns with CC genotype was found, therefore lactose intolerant, being these data in accordance with the literature [1,3,4,13,23-25], including national data [20-22]. From these lactose intolerant newborns, 32% (1:1.96) are male and 30% (1:2.03) are female, not being the relation between genders statistically significant (p=0.801). Due to the inheritance that determines the lactose intolerance not being linked to the sexual chromosomes, the results should not really demonstrate the significant difference between genders [26,27].

Concerning the Brazilian ethnic, a prevalence of 53% of lactose intolerance was identified in white individuals and 91% in non-white ones by the measurement of lactase activity in the duodenal mucosa by imunohistochemistry [28]. But, recently, the genetic test was correlated with the Brazilian ethnic being the prevalence of about 57% of intolerance for the white color group, 80% for the black color group and 100% of lactose intolerance for Japanese descendants [29], confirming previous data about the ethnic prevalence of lactose intolerance diagnosed by conventional methods [8-12].

Up to the moment, there are no data in the international or Brazilian literature on neonatal screening of lactose intolerance, as carried out in the present study. It is probably, due to the fact of recent discoveries of molecular bases of lactose intolerance and that the studies were based, primarily, in comparing whether the diagnosis obtained by means of conventional complementary exams, as the expired H_2 test, confirmed the molecular diagnosis.

Recent studies have demonstrated an agreement from 91 to 97% of the CC genotype with positive results of the expired H_2 test and from 86 to 95% of agreement between the CT and TT genotypes and negative results of the expired H_2 test, besides having presented positive predicting values of 97% and negative of 86% for the molecular test [1,22,30-32]. All these studies that compared the polymorphism presence and the expired H_2 test were performed in the European countries, except one study which carried out the molecular analysis of lactose intolerance in a non-European population, also demonstrating, a significant agreement between genotyping and the expired H_2 test results [33].

The lactase enzyme expression analysis was also performed in patients with the diagnosis of lactose intolerance. In this study, it was determined that the correlation of the lactase enzyme expression is much higher in patients with T allele than in those with C alleles, suggesting that this polymorphism is related to the lactase gene transcription regulation, therefore, to the non-persistence of the lactase enzyme in the intestinal mucosa [16].

Another positive correlation was carried out in a study, with children, in which the genetic material was obtained by intestinal biopsy. Despite the positive correlation, the way the genetic material was obtained, created certain rejection from parents, because the biopsy is an invasive test. The authors conclude, that the biopsy is not the diagnostic procedure of first choice, mainly in children, unless there is no access to molecular tests [24].

In Brazil, there are few studies comparing the polymorphism presence and the expired H_2 test, obtaining a correlation in adult patients with suspicion of lactose malabsorption or with irritable colon syndrome in about 96% of cases. In these, considering the -13910C/T polymorphism as a diagnostic test for lactose intolerance, the CC genotype presence was estimated to have a sensitivity of 100%, specificity of 83%, positive predictive value of 76% and negative predictive value of 100% [20-22]. Another study was carried out in Brazil, specifically in children under lactose intolerance suspicion, was performed by means of expired H2 test without the genetic test, being the prevalence of 84% of lactose malabsorption in this Brazilian pediatric population [34].

The results from these studies also show that, in the individuals with the positive expired H2 test and with the negative genetic test, which are good reasons to suspect of secondary causes of lactase deficiency. The high prevalence of the lactase non-persistence in the general population, should convince the doctors into being restrictive, when the diagnosis of primary lactose intolerance is based only on the expired H2 test [4,22-24,31,33].

Thus, the genetic tests PCR/RFLP, to analyse the polymorphism of an only nucleotide -13910C/T located upstream of the lactase gene, can be considered a great analysis in predicting the lactose intolerance in a population under the suspicion of malabsorption, differentiating patients with primary lactose intolerance from those with secondary intolerance. It is a simple molecular test, non-invasive and comfortable exam, besides it is faster than the lactose tolerance test and does not lead to the symptoms of intolerance to this sugar, such as diarrhea and abdominal pain. Additional advantages of PCR/RFLP, when compared to the expired hydrogen test are that the molecular test is less expensive, cause less discomfort, since the venous blood sample can easily be sent to the laboratory, does not ask for fasting, dietary preparation, stimulus with lactose and takes less time from the patient.

The incorrect diagnosis of lactose intolerance in CC-negative patients can result in a long and lasting restriction of dairy products. In these cases, it would not relieve the symptoms and would even contribute to osteoporosis and other related complications. Therefore, each patient with a complaint related to lactose consumption should be first investigated by the genetic test and, subsequently, by the additional procedures in order to clarify whether the symptoms are related to other gastrointestinal conditions. Since, the costs of the genetic test do not exceed those of the expired hydrogen test, this strategy can also be cost effective. In cases of lactase secondary deficiency, accurate methods of

diagnosis and therapy will lead to a decrease of lactose-related complaints, being able to restore the lactase activity [31].

Adequate dietary recommendations are necessary for lactose intolerant, since they tend to present a decrease in the levels of calcium due to the low intake of milk and to the impairment in its absorption. Pediatricians and nutritionists, specialized in infantile dieting, should bear the benefits in mind and controversies related to the consumption of milk and its derivatives and of infant formulas which have lactose [35]. It should be noticed that, if parents get the diagnosis that their child has a CC genotype, still in the stage in which the lactase activity is high and/or when no symptoms of lactose intolerance have manifested, this fact can have an undesirable effect in relation to the dietary aspect and, therefore, nutritional of the child [24,31,35].

CONCLUSIONS

In summary, the molecular analysis identified the presence or the absence of the variable lactase persistence in Brazilian newborns. The neonatal molecular diagnosis can optimize the follow-up of positive results in newborn screening for lactose intolerance. These results strengthen the genetic test applicability for the -13910C/T polymorphism in the lactase non-persistence diagnosis, making it available not only for individual diagnostic purposes, but also, as a mean of screening for population study and/or neonatal, as carried out in the present study.

CONFLICT OF INTEREST

There are no financial bounds or agreements between the authors and companies that may be interested in the material addressed in this Article.

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