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Neonatal detection of Turner syndrome by real-time PCR gene quantification of the *ARSE* and *MAGEH1* genes

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ABSTRACT. Turner syndrome (TS) is characterized by the presence of one full X chromosome and total or partial deletion of the second sex chromosome. Diagnosis of TS is often delayed, resulting in inappropriate treatment. Early diagnosis of TS using a neonatal screening test may improve preventive measures and treatment, thus improving patient quality of life. The goal of this study was to standardize a neonatal TS screening algorithm. Two study genes (*ARSE* and *MAGEH1*) and 1 normalizing gene (*HBB*) were used to detect the second X chromosome. We screened 996 newborns whose peripheral blood was collected and stored in filter paper. In addition, samples from 20 patients with

confirmed diagnosis of TS were included in the study. Relative amounts of ARSE/HBB were determined using real-time polymerase chain reaction. The cutoff at the 5th percentile was arbitrarily set to indicate repetition of the test. The test was repeated in 51/1016 patients with ARSE/HBB < 0.81. For 10 samples with values persistently <0.81, we quantified the MAGEH1/HBB ratio. Values below the 95th percentile in TS patients (MAGEH1/HBB < 1.24) were considered to be inadequate. Only 6/996 NB showed inadequate values for the 2 studied genes, which were recalled for clinical evaluation and karyotype testing. Analysis of 20 patients diagnosed with TS allowed for identification of false-negatives and true-positives, establishing 95% sensitivity when the indicated cutoff values were used. In conclusion, our algorithm reached 95% detection sensitivity with an acceptable recall rate (0.6%), allowing for the detection of suspected TS cases in the neonatal period.

Key words: Neonatal screening; Real-time polymerase chain reaction; Turner syndrome

INTRODUCTION

Turner syndrome (TS) is a common chromosomal abnormality present in 3% of conceptions and in 1 in 2500 live female births. The syndrome is characterized by the presence of 1 full X chromosome and total or partial deletion of the second sex chromosome (Gravholt et al., 1996; Cardoso and Piazza, 2000).

The main clinical features of TS include smaller size than expected for the gestational age, lymphedema of the hands and feet, redundant posterior cervical skin, and low hair implantation. Diagnosis is often delayed, typically occurring around the age of 15; fewer than 20% of the cases are diagnosed during the neonatal period. Not only the neonatal detection of TS potentially offer the advantages associated with early diagnosis, such as identification of heart and kidney anomalies, but it also allows for the prevention and more efficient treatment of short stature and gonadal dysgenesis (Gil del Alamo and Cano Rodríguez, 2001; Sybert and McCauley, 2004; Stratakis and Rennert, 2005).

Karyotyping is considered to be the gold-standard cytogenetic technique for diagnosing TS. This method can be used to detect chromosome abnormalities such as translocations, deletions, inversions, duplications, and mosaicisms. Karyotyping for neonatal screening has important limitations, such as running time, cost, and need for specialized personnel, among others (Fröhling et al., 2002; Siegel and Sybert, 2005; Klein, 2011). Therefore, various molecular methods have been proposed for diagnosis or neonatal screening of TS, including Southern blotting, polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP), fluorescent PCR genotyping, GeneScan-based genotyping, pyrosequencing, and real-time PCR (Gicquel et al., 1998; Longui et al., 2002; Cirigliano et al., 2004; Meng et al., 2005; Figueiredo et al., 2008; Rocha et al., 2010; Rivkees et al., 2011; Aksglaede et al., 2012).

The goal of this study was to standardize a diagnostic algorithm for TS by identifying the relative copy number of genes present in the short arm of the X chromosome in relation to an autosomal control gene. This allowed for the identification of a gene deletion compatible with TS.

MATERIAL AND METHODS

Individuals

Peripheral blood samples were collected from the heels of 996 newborns (NB) at the Maternity Department at Irmandade da Santa Casa de Misericórdia de São Paulo from November 2010 to June 2012. Samples were obtained by following the general collection guidelines of the neonatal screening programs at the same time samples for other classical screening tests were collected; the samples were stored in filter paper and numerically and anonymously identified. Patients' personal and clinical data were stored in a separate database, blind to the investigator who conducted the laboratory tests. At the Pediatric Endocrinology outpatient clinic, samples from 20 patients diagnosed with TS were also collected, among which 10 had the 45,X karyotype and 10 presented various forms of X mosaicism. These samples were randomly inserted among the other samples in a blinded fashion and provided to the investigator. All research subjects' parents/guardians signed a voluntary informed consent form agreeing to the patient's inclusion in the study. The study was approved by the Institution's Research Ethics Committee (protocol No. 316/10).

Molecular analysis

DNA was extracted from the samples by puncturing 3 filter paper disks that were 2 mm in diameter each, which were then transferred into a 1.7-mL microtube. The blood present in the disks was eluted by incubation at 60°C for 30 min with 180 µL MGM Pureplus purification reagent (Catalogue No. 23020100; MGM Assessoria Biológica; Curitiba, Paraná, Brazil) and 0.7 µL proteinase K. The sample was then homogenized by vortexing. Next, a second elution was performed with 180 µL MGM Pureplus reagent, with the incubation time reduced to 15 min. A series of 3 elutions with UltraPure distilled water (Catalogue No. 10977-015; Gibco; Invitrogen; Carlsbad, CA, USA), each for 5 min at 60°C, was conducted to remove red blood cells. For the final DNA elution, 30 µL UltraPure distilled water was added to the tube, which was incubated at 60°C for 30 min. The supernatant containing DNA in suspension was then recovered, transferred into a new microtube, and stored at -20°C for further amplification by real-time PCR.

Genomic DNA from a control female patient with a 46,XX karyotype was used to prepare a standard curve and establish the quantitative parameters of a normal sample. After initial 1:100 dilution of the stock solution, subsequent points on the standard curve were established using serial 1:1 dilutions of genomic DNA in UltraPure distilled water. All real-time PCRs used the same standard curve, allowing for control of inter-assay variations and the establishment of reproducible cutoffs for different assays.

The amount of genetic material in the short arm of the X chromosome was determined using 2 genes of interest, the *ARSE* gene (arylsulfatase E-OMIM 300180) and the *MAGEH1* gene (melanoma antigen, H1-OMIM protein: 300548). The former is located in the telomeric pseudoautosomal region (ARSE-Xp22.3), while the latter is in the pericentromeric region (MAGEH1-Xp11.21), allowing for detection of partial or total deletion of the short arm of the X chromosome. The following primers were used: *ARSE* sense primer: TTG TGA CGC CTG TGT TCCA (Invitrogen); anti-sense primer: GGC AGA CCT TTC TTC CAT AGCA (Invitrogen); probe: 6-FAM- CCA GAG GGA GCC GGT-MGB (Applied Biosystems; Foster City, CA, USA); *MAGEH1*, assay Hs01183540_cn-FAM (catalog No. 4400291; Applied Biosystems).

To ensure that the same amount of DNA was used in each reaction, genes of interest were normalized to beta hemoglobin (*HBB*, 11p15.5, OMIM OMIM 141900), which is present as a single copy gene in the autosomal chromosome. The selected normalizing gene, when in duplex, had the advantage of being amplified as efficiently as were the genes of interest. The sequences used for the amplification of the normalizing gene were: sense primer: GTG CAT CTG ACT CCT GAG GAG A (Invitrogen); anti-sense primer: CCT TGA TAC CAA CCT GCC CAG (Invitrogen); probe: AAG GTG AAC GTG GAT GAA GTT GGT GG-TAMRA (Applied Biosystems). PCRs were carried out in a 7500 Real-Time PCR System (Applied Biosystems), and the parameter setting was conducted with the aid of the SDS-Sequence Detection System version 1.2, 7500 Systems SDS Software (Applied Biosystems). The thermocycling conditions used were 50°C for 2 min; 95°C for 10 min; 40 cycles at 95°C for 15 s; and 60°C for 1 min; fluorescence readings were recorded during the final step of each cycle (Rocha et al., 2010).

Reactions were performed in duplicate and in duplex (gene of interest and normalizing gene in the same tube), with average quantities expressed as a simple ratio of the gene of interest to the normalizing gene (*ARSE*/*HBB*; *MAGEH1*/*HBB*).

A pre-established algorithm was used to sequentially interpret the results (Figure 1). For the 996 samples collected, the 5th percentile (p5) was calculated for the *ARSE/HBB* quantity, which was then considered to be the cutoff value for defining any given sample as either normal or as a candidate for repeated PCR. Therefore, samples whose results were below p5 were again quantified to determine the *ARSE*/*HBB* ratio. Samples with values persistently below p5 were submitted for quantification of the second study gene *MAGEH1*/*HBB*. The cutoff for this second gene was defined as the p95 of patients diagnosed with TS, as confirmed by karyotyping. Values below this cutoff were considered to indicate a TS diagnosis. Newborns with inadequate values for both study genes were recalled for a clinical evaluation, repeated round of molecular analysis, and karyotyping. The molecular analysis conducted for the 20 patients with a TS diagnosis confirmed by karyotyping allowed for the identification of false-negative and true-positive cases. This enabled identification of the sensitivity of the cutoffs established for each gene of interest.

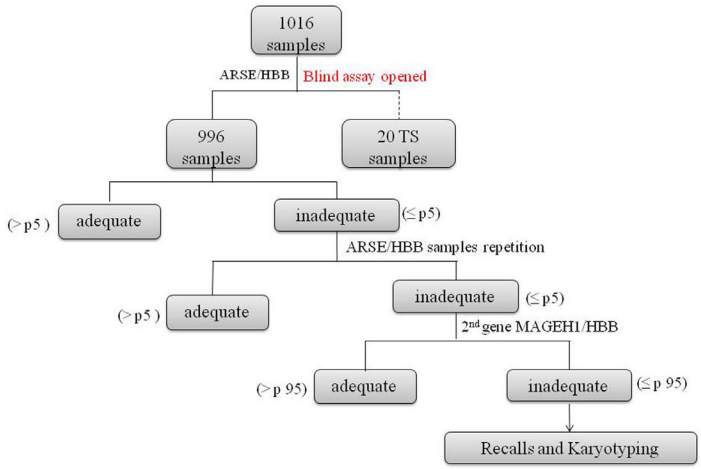

Figure 1. Algorithm of analysis for the genes ARSE and MAGEH1 in samples of newborn girls and of patients with Turner syndrome.

RESULTS

The distribution of the *ARSE*/*HBB* and *MAGEH1*/*HBB* ratio results for the NB and patients with TS are shown in Figure 2. The 5th percentile, which was arbitrarily defined as the cutoff for the *ARSE/HBB* ratio, was 0.81. The 51 samples showing results below 0.81 were submitted to a new round of analysis for the *ARSE* gene. Ten samples showed persistent results below p5; therefore, these samples were analyzed for the second study gene (*MAGEH1*).

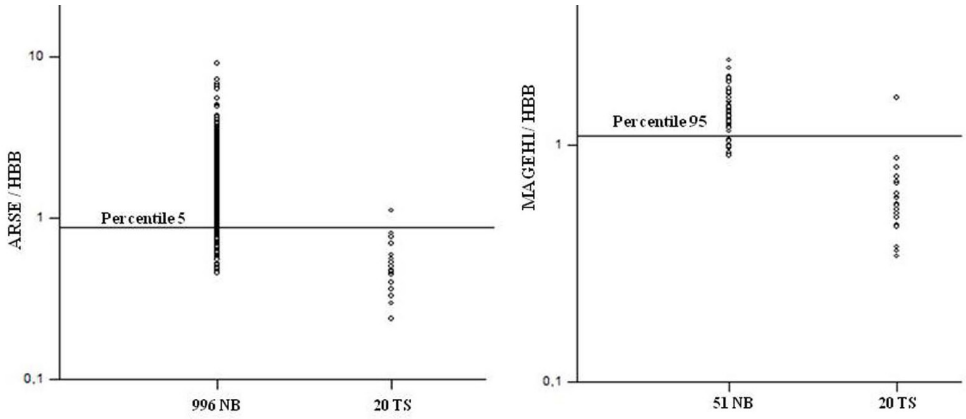

Figure 2. Graphic representation of distributions of ARSE/HBB and MAGEH1/HBB ratios in newborn. The corresponding values for the 5th percentile and the values for the 95th percentile of patients with Turner syndrome are shown, respectively.

For the *MAGEH1/HBB* ratio, the 95th percentile was arbitrarily defined as a cutoff value of 1.24. Among the 10 samples from NB patients evaluated for the *MAGEH1* gene, 6 were found to have values below or equal to 1.24. Because of a suspected TS diagnosis, these samples were recalled for karyotyping. Global results obtained using the proposed algorithm are shown in Figure 3.

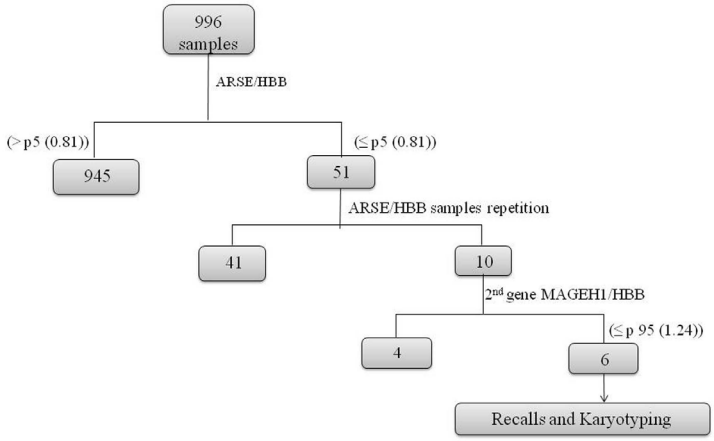

Figure 3. Results obtained by employing the proposed algorithm for identifying suspected cases for Turner syndrome.

In the protocol proposed, the repetition rate of the PCR experiment was 51/996 (5%) for the analyses involving the *ARSE* gene, and 10/996 (1%) for those involving the *MAGEH1* gene. The patients' recall rate was 6/996 (0.6%).

Among those diagnosed with TS, only 1 patient showed a false-negative result in the first round of the *ARSE* gene analysis, i.e., *ARSE*/*HBB* ratio > 0.81. When detecting the *MAGEH1* gene in the remaining 19 patients with TS, only 1 showed results above the p95 (>1.24); this patient was thus considered to be a false-negative for the *MAGEH1* gene. Therefore, the test sensitivity obtained was 95% with a positive predictive value of 1 in the analyses for both the *ARSE* and *MAGEH1* genes.

Only 2/6 newborns responded to the recall request. Molecular tests were repeated following collection of new samples. The subject identified as NB 723 presented an *ARSE/HBB* ratio result of 0.7 (below desirable) and a *MAGEH1/HBB* ratio of 0.59 (below desirable). The subject identified as NB 732 presented an *ARSE/HBB* ratio result of 0.68 (below desirable) and a *MAGEH1/HBB* ratio of 0.74 (below desirable), confirming the results obtained in the first molecular analysis. Despite these altered molecular values, the karyotype of both patients was 46,XX in 50 metaphases analyzed. In these 2 cases, further studies using the multiplex ligation-dependent probe amplification technique and genetic sequencing will be necessary to understand the cause of the discrepancy in results between the karyotyping analysis and molecular findings.

DISCUSSION

The goal of this study was to establish a standardized real-time PCR protocol as a neonatal screening algorithm for detecting TS. In order for a laboratory test to be considered viable for population screening of live NBs, it must rely on techniques that present low recall rates, allow for safe confirmation of the diagnosis, and ensure that the appropriate care and treatment are given to the patients diagnosed (NUPAD, 2013).

For TS, a neonatal screening test must have a profile similar to that of established programs, such as congenital hypothyroidism and cystic fibrosis detection methods, potentially improving the quality of life of the patients.

Because karyotyping is both a high-cost technique and demands a considerable amount of time for its completion, its application is restricted to the confirmation of TS in individuals who have been recalled in the neonatal screening program.

Other molecular methods have been proposed for detecting TS. Monsen (2010) described the importance of developing molecular methods for neonatal screening using microarrays when studying chromosomal abnormalities associated with rare anomalies. This technique, as well as karyotyping, has a high cost when used on a large scale. By contrast, the real-time PCR technique used in our study not only has lower costs, but also has the potential to be automated, effectively used on a large scale, and easily adjusted to neonatal screening programs (Tsé and Capeau, 2003).

Gicquel et al. (1998) pioneered a study protocol for identifying patients with TS using Southern blotting after evaluating 375 DNA samples from girls of low stature. The protocol showed 94.7% sensitivity in the detection of TS, which is very close to the sensitivity obtained in our study, which was 95% for each of the genes studied. In addition, the real-time PCR technique shows greater reproducibility, higher speed, and higher efficiency when compared with Southern blotting (Tsé and Capeau, 2003; Bustin et al., 2009).

Our group previously proposed the use of PCR-RFLP (Longui et al., 2002) after evaluating the coding gene for the human androgen receptor (AR) (HUMANARA - OMIM 313700). This highly polymorphic gene allows for the determination of the presence or absence of the second X chromosome. Among the 22 patients with TS (18 cases 45,X; and 4 cases 45,X/46,XX), we appropriately identified the 45,X patients. In addition, we identified 3 patients with low frequency 45X/46XX mosaicism not previously identified using traditional karyotyping techniques. The PCR-RFLP technique appears to be useful for studying isolated groups, but has some limitations related to the long period of time needed for its set-up, preventing its wide use in neonatal screening programs.

Cirigliano et al. (2004) presented an analysis of 18,000 samples (81 of which were samples from TS patients) evaluated using fluorescent PCR genotyping. The test permitted the detection of 55 of 56 cases with the 45,X karyotype and 12 of 25 cases of mosaicisms.

Our group (Figueiredo et al., 2008) also proposed the use of genotyping employing GeneScan for assessing the repetition number of CAG in the AR exon 1. In a group of 30 girls with short stature and suspected TS, 9 patients were homozygous and 21 were heterozygous. Among the 9 homozygous patients, 6 had TS and a 45,X karyotype. In the heterozygous group, 17 patients presented with a normal 46,XX karyotype, whereas 4 patients were diagnosed as carrying TS associated with a form of chromosomal mosaicism. The sensitivity of the test was 100% in the 45,X cases. However, as reported by Cirigliano et al. (2004), the sensitivity reached by the test proposed was inadequate for detecting mosaicisms. Therefore, genotyping also represents an efficient alternative method for identifying patients with TS, particularly among a specific group of patients with short stature. Nevertheless, this method is not entirely suitable for a neonatal screening program considering that it is both costly and time-consuming.

Meng et al. (2005) used pyrosequencing coupled with the analysis of single-nucleotide polymorphisms regions on the X chromosome. The method showed 100% sensitivity and specificity, both for the 45,X and X mosaic samples. Nevertheless, its cost is high because of the large number of chromosomal regions that must be analyzed. Because of the high number of samples involved in population screening, the use of this technique is impracticable. Rivkees et al. (2011) also used pyrosequencing to screen for TS. The authors performed relative quantification of alleles, selecting 18 single-nucleotide polymorphism regions for the X chromosome and 1 for the Y chromosome. Analysis of peripheral blood DNA reached 96% sensitivity in patients with TS.

We previously studied the real-time PCR technique in the identification of TS (Rocha et al., 2010) by quantifying the *ARSE/GAPDH* genes ratio. In our previous study, we obtained 100% sensitivity and specificity for TS patients and the 45,X karyotype, but only 56% sensitivity in patients with mosaicism.

In the present study, we used the *ARSE* gene, which is located in the telomeric pseudoautosomal region of the short arm of the X chromosome, and added a second gene of interest, *MAGEH1*, which is located in the pericentromeric region of the short arm of the X chromosome. The latter gene was chosen to aid in the identification, both for cases in which the short arm deletion is complete and in those of partial deletions and mosaicism of the X chromosome.

The test proposed in this study detected TS in 18 of 20 patients with this syndrome who were used as internal controls for identifying false-negative cases that had been added in a random and blind fashion to the samples collected from the NB. The 2 cases in which detection failed had an X mosaic karyotype.

The 2 TS patients whose samples were initially identified as false-negatives during

the first round of analysis of the *ARSE*/*HBB* and *MAGEH1*/*HBB* genes showed results below the cutoff established for both genes of interest that could allow for the identification of the cases using 3 other methods for the same samples. This finding suggests the presence of some interfering agent during the first reaction, which may have led to the false-negative result.

Our results confirmed that the real-time PCR protocol proposed in this study is an efficient method for identifying insufficient gene copies in the short arm of the X chromosome. In addition, this protocol may be applicable for population screening of patients with TS because its sensitivity is 95% and the predicted positive value is 1. Further analysis of the results from patient recalls, including karyotyping of all of the cases suspected, will contribute to the recognition of false-positive cases.

Using a similar method as the one developed in this study, Aksglaede et al. (2012) proposed molecular identification of Klinefelter syndrome in 50 patients using genomic DNA evaluated by real-time PCR for the *SHOX* (OMIM: 312865) and *AR* genes. By comparing the quantities of the genes of interest and the normalizing gene *GAPDH* (OMIM 138400), the authors found 100% sensitivity in detecting Klinefelter syndrome. These findings corroborate the potential applicability of real-time PCR in neonatal screening programs.

Because we did not conduct a clinical or laboratory follow-up on NB whose values were considered normal, our study is limited because we did not calculate the negative predictive value for the normal test.

In addition, the financial viability of the tests proposed for large-scale neonatal screening limits the use of this technique. In this study, the cost of each real-time PCR test was approximately US $15.00. The Brazilian Unified Health System establishes a maximum cost of US $20.00 for conducting molecular tests for conditions investigated under a neonatal screening routine (NUPAD, 2013; SUS, 2013). These costs may be further reduced if the tests are conducted on a large scale.

In summary, using the cutoffs proposed in our study and with 5% of the total samples undergoing repeated evaluation, 1% of the initial samples showed consistently low results, requiring the analysis of a second gene. The sequential results combined for the 2 genes yielded an estimated 0.5% patient recall rate, which is compatible with recall rates observed in programs already established for other diseases.

In the present study, conventional neonatal screening conditions were used to develop a real-time PCR technique for detecting TS. Future studies involving a representative number of patients from the population conducted in different urban centers are needed to confirm the actual recall rates and the efficacy of our method in identifying pathological cases. Thus, our neonatal screening algorithm, involving the relative quantification of the *ARSE*/*HBB* and *MAGEH1*/*HBB* genes using real-time PCR in DNA samples collected from peripheral blood and stored on filter paper, respectively yielding 0.81 and 1.25 cutoffs, is a suitable method for identifying patients with a suspected TS diagnosis. Therefore, this algorithm may be applied for neonatal screening programs to identify patients with TS.

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