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Development of a TaqMan-based dosage analysis PCR assay for the molecular diagnosis of 22q11.2 deletion syndrome

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A hemizygous 1.5–3.0-Mb microdeletion of human chromosome 22q11.2 with the loss of multiple genes including histone cell cycle regulator (*HIRA*) causes 22q11.2 deletion syndrome (22q11.2 DS), a common disorder with variable manifestations including congenital malformations affecting the heart, palate and kidneys in association with neurodevelopmental, psychiatric, endocrine and autoimmune abnormalities. The aim of this study was to develop a TaqMan-based dosage analysis PCR (TaqMan qPCR) for use as a rapid, cost-effective test for clinically suspected patients fulfilling previously described criteria for molecular diagnosis of 22q11.2 DS in a lower middle-income country where the cost of testing limits its use in routine clinical practice. Nineteen patients were recruited with informed consent following ethical approval from the Ethics Review Committee, Lady Ridgeway Hospital for Children, Colombo. Dosage analysis of extracted DNA was performed using a TaqMan qPCR assay by amplifying regions within the target (*HIRA*) and control (testin LIM domain protein (*TES*)) genes of suspected patient (P) and unaffected person (N) samples. For detection of a deletion, the normalized value (*HIRA*/*TES* dosage) of a P sample was compared with that of an N sample. A ratio of P:N of 0.5 confirmed the presence of a deletion while a ratio of 1.0 refuted this. Seven of the 19 patients were found to have a *HIRA* deletion, confirming the diagnosis of 22q11.2 DS, with these results being in complete agreement with those of fluorescence *in situ* hybridization (FISH) (performed in nine of the 19 cases) and whole-exome sequencing (all 19 samples tested). This TaqMan qPCR assay was able to reliably distinguish *HIRA*-deleted cases from non-deleted ones. The assay was both cheaper and faster compared to commercially available alternatives in our setting, including FISH and multiple ligation-dependent probe amplification.

Key words: TaqMan quantitative PCR, 22q11.2 deletion syndrome, fluorescence *in situ* hybridization, whole-exome sequencing

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Chromosome 22q11.2 deletion syndrome (22q11.2 DS), also referred to as velocardiofacial syndrome or DiGeorge syndrome, is a common microdeletion syndrome (Zinkstok et al., 2019) with variable, multisystem involvement (Hestand et al., 2016). Around 90% of 22q11.2 DS patients have a 3-Mb microdeletion in chromosome 22q11.2. This consists of a typically deleted region (TDR) containing around 90 genes, including 46 protein-coding genes (Hestand et al., 2016; Zinkstok et al., 2019). Around 5% of

22q11.2 DS-affected patients have a nested 1.5-Mb deletion (encompassing 28 genes), including the histone cell cycle regulator gene *HIRA* (also called *TUPLE1*), within the TDR, while fewer than 5% have smaller or atypical (outside the TDR) deletions (Zinkstok et al., 2019). Clinical suspicion of this syndrome is confirmed by detecting the 22q11.2 microdeletion (McDonald-McGinn et al., 2020).

The traditional gold standard method for confirming the 22q11.2 microdeletion is fluorescence *in situ* hybridization (FISH) (Hwang et al., 2014). More recently, multiplex ligation-dependent probe amplification (MLPA) and array comparative genomic hybridization have also been widely used for diagnosing 22q11.2 DS (McDonald-McGinn et al., 2020). The *HIRA* gene within the TDR (Zinkstok et al., 2019) is the target of the Vysis TUPLE 1 (HIRA) FISH probe (see below), which detects around 95% of 22q11.2 DS patients (Chen et al., 2006).

The main problem in confirming 22q11.2 DS in low and middle income countries is the cost of testing (Hwang et al., 2014). Previous studies have described the use of quantitative PCR (qPCR) for the rapid diagnosis of 22q11.2 DS. Chen et al. (2006) described a less expensive qPCR method using a SYBR Green I detection system. This real-time PCR method assessed and validated 122 patients suspected of having DiGeorge syndrome. Within this cohort, 28 patients were found to have a deletion, which was later confirmed through FISH analysis. This methodology demonstrated high reliability and was both time-efficient and cost-effective for the detection of 22q11.2 deletions (Chen et al., 2006).

A TaqMan PCR-based gene dosage analysis method for diagnosing 22q11.2 DS using *UFD1L* as the target and S100 calcium-binding protein B on chromosome 21 as the control region was described in a study of 29 confirmed patients, which reported that the method was rapid and accurate in diagnosis of 22q11.2 DS (Kariyazono et al., 2001). More recently, Campos-Garcia et al. (2022) described a TaqMan qPCR screening method to identify 22q11.2 DS in patients with congenital conotruncal heart defects. Their study, involving 23 patients, used a three-probe approach targeting *TBX1*, with RNase P as an internal reference. Of the 23 patients, 21 tested negative while two exhibited monoallelic copy number predictions. One of these cases was subsequently confirmed using MLPA analysis.

We targeted *HIRA* for the development of a cost-effective TaqMan qPCR assay for the diagnosis of 22q11.2 DS. Although TaqMan qPCR assays are more expensive than SYBR Green I systems, the TaqMan assay we developed remained affordable for use in Sri Lanka because, apart from the initial cost of probes, many of the reagents used were prepared in-house.

Nineteen suspected 22q11.2 DS patients were recruited with the informed consent of their parents following approval from the Ethics Review Committee of Lady Ridgeway Hospital for Children, Colombo. Inclusion criteria were the presence of orofacial clefting with or without conotruncal congenital heart defects plus developmental delay/learning difficulties. All cases also needed to fulfill previously described criteria for 22q11 deletion testing (Monteiro et al., 2013).

Whole blood leukocytes of patients (P) and unaffected individuals (N) were used for DNA extraction using the QIAamp Blood Mini Kit (QIAGEN, Germany). Extracted DNA was quantified spectrophotometrically.

Molecular diagnostic testing was performed for identifying a deletion of *HIRA* located in chromosome 22q11.2. A 164-bp target region in *HIRA* was amplified and quantified, together with a non-deleted control region (a 206-bp region within the testin LIM domain protein gene) on chromosome 7, in a duplex TaqMan qPCR assay. The control was used to normalize sample-to-sample variation.

For detection of a deletion, the P and N samples are amplified and quantified, and the ratio of the normalized value of the P sample (*HIRA/TES* dosage) to that of an N sample is determined. A patient with a deletion is expected to have a P:N ratio of 0.5, while an individual without a deletion is expected to have a ratio of 1.0.

The primers and TaqMan probes for *HIRA* and *TES* (Table 1) were designed using the online primer and probe design software tool OligoArchitect (Merck, USA). They were verified using BLAST (NCBI), BLAT (UCSC Genome Browser) and the OligoAnalyzer tool (Integrated

Table 1. Sequences of primers for amplification of 22q11.2 DS target and control regions

Primer	Sequence (5′→3′)	T_m (°C)
HIRAF	GGAGATCACAAATGACTCTC	59.5
HIRAR	GCTTCAGAAACAAAGACCTA	59.6
HIRAprobe	FAM-TCTGCTAGACGGTTATGACTGAACAAC-BHQ1	67.6
TESF	GTTCCAAATCCTTGAACTGA	59.9
TESR	CTGAGGCATCTAATCATCTG	59.4
TESprobe	HEX-CCTCAGTTCTTGTGACACTCTGCC-BHQ1	68.4

T_m, melting temperature.

DNA Technologies, USA), and custom-synthesized by and purchased from Macrogen, South Korea. The *HIRA* probe was labeled with the FAM reporter dye and BHQ1 quencher; the *TES* probe was labeled with the HEX reporter dye and BHQ1.

The optimized reaction mixture for *HIRA* and *TES* TaqMan duplex PCR contained 50 ng of extracted genomic DNA, 1×PCR mix [$MgCl_2$ (2 mM), 200 µM dNTPs (Promega, USA)], 1×PCR buffer, primers HIRAF/R (0.6 µM each), TESF (0.4 µM) and TESR (0.3 µM), 0.25 µM each of HIRAprobe and TESprobe and 1 U of *Taq* DNA polymerase (UC Biotech, Sri Lanka) in a final reaction volume of 25 µl. A two-fold DNA dilution series (200, 100, 50, 25 and 12.5 ng) and a 10-fold dilution series (100, 10, 1 and 0.1 ng) were also included in each assay.

TaqMan-based dosage analysis PCR was carried out with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA), and the amplification profile of a typical PCR reaction was a 10-min initial denaturation at 95 ºC followed by 35 cycles of 15 s at 94 ºC, 30 s at 60 ºC and 1 min at 72 ºC. Included in each assay was a positive control, normal control and no-template control. All samples were analyzed in duplicate. Serially diluted DNA was used to generate a standard curve to quantify the amplified DNA. TaqMan qPCR was also carried out to analyze DNA samples (n = 9) from non-deleted/unaffected individuals.

Based on the quantification cycle, the DNA copy numbers of each gene were obtained from the standard (calibration) curve and the *HIRA* copy number was normalized using the *TES* gene.

FISH was performed using metaphase spreads prepared from lymphocyte cultures of nine of the 19 patients using optimized protocols (Fauzdar et al., 2012). The Vysis commercial FISH probe set (Abbott, USA) consisted of a gene-specific probe, 117 kb SpectrumOrange LSI TUPLE 1 (HIRA) (encompassing *HIRA* and D22S553 on chromosome 22q11.2), and a control probe, 334 kb SpectrumGreen ARSA (located on chromosome 22q13.3). The slides were observed under a BX53 fluorescence microscope (Olympus, Japan).

Whole-exome sequencing (WES, performed at Macrogen) results (n = 19) for copy number variations (CNVs) were obtained from a parallel study. Exome capture libraries were generated using Agilent SureSelect^{XT} Low Input protocols for Illumina paired-end sequencing libraries with 1.0 µg of input gDNA. CNVs were detected using read count data extracted from WES data using recalibrated BAM files. Five to ten WES datasets from Sri Lankan individuals (gender-matched to the patients) were used as a reference set. Detection of CNVs utilized the ExomeDepth (Plagnol et al., 2012) and CNVkit (Talevich et al., 2016) tools. Results from both tools were compared to identify CNVs in the selected chromosome regions.

Nineteen patient samples were analyzed by TaqMan qPCR and also subjected to WES (n = 19 cases) and FISH analysis (n = 9 cases). Seven of the 19 cases (37%) were confirmed to have the *HIRA* target region deletion (Table 2).

The mean corrected *HIRA* dosage of deleted patients was 0.50 ± 2 SD (range 0.40–0.60) while that of unaffected controls was 1.04 ± 2 SD (range 0.76–1.32). The TaqMan qPCR assay was able to clearly detect the deletion with a statistical confidence level of 95% with a P-value of 0.05. There were no overlaps in the gene dosages between patients with deletions and unaffected controls. The results were in complete agreement with those of FISH and WES (Table 2).

Identification of the molecular basis for 22q11.2 DS has enabled the development of diagnostic tests (Shprintzen, 2000) with or without the use of clinical criteria for testing (Monteiro et al., 2013). Among the methods used, FISH is reliable, but labor-intensive and expensive. Occasional false negative results may occur due to the target probe being limited to a single region within 22q11.2 (Stachon et al., 2007). The other commonly used method, MLPA, remains expensive and not widely available in low and middle income countries.

Dye-based detection involves introducing a DNA-binding dye such as SYBR Green I or EvaGreen (Arya et al., 2005) into the PCR. The SYBR Green family of dyes bind double-stranded DNA amplified during PCR in a non-specific manner. Though it is less expensive, its non-specific DNA binding limits its use in qPCR (Arya et al., 2005). Chen et al. (2006) performed a study on 122 suspected 22q11.2 DS patients using SYBR Green I, and identified 28 patients with a chromosome 22q11.2 deletion. Although we initially used SYBR Green dye for our samples, ambiguous results were obtained.

Therefore, we developed a TaqMan-based dosage analysis PCR assay for 22q11.2 DS diagnosis. Fluorophore-coupled nucleic acid probes (TaqMan probes) allow the quantification of a sequence of interest during qPCR (Arya et al., 2005). The assay also allows for multiplexing.

In our study, TaqMan probes were developed for *HIRA* (target region in chromosome 22) and *TES* (control region in chromosome 7). The deletion of one copy of *HIRA* was clearly detected by TaqMan qPCR with a statistical confidence of 95%. Seven of the 19 patients tested were found to have the deletion while 12 tested negative. These results were confirmed using WES in all 19 investigated patients, and using FISH in 9/19 patients. Due to financial restrictions the gold standard method, FISH, could only be used in 47.3% of the patients studied. Although WES is not a gold standard method, it is accepted universally as a reliable and accurate method for CNV diagnosis.

Our TaqMan qPCR assay (costing around 26 USD) was found to be less expensive than FISH (around 109 USD) and MLPA (around 99 USD), which are the other currently available tests in Sri Lanka.

This test was developed as a part of a research study, and validation of results using WES was only possible for

Table 2. Results of exome sequencing/CNV analysis, TaqMan qPCR and FISH

No.	Patient			Exome sequencing/CNV analysis		TaqMan qPCR	FISH
	ID[1]	Gender[2] F/M	Age (months)	D/ND[3]	Size of deletion (Mb)		
1	VCFS2	M	168	ND	-	ND	
2	VCFS10	F	170	ND	-	ND	ND
3	VCFS18	F	128	ND	-	ND	
4	VCFS26	F	143	ND	-	ND	ND
5	VCFS27	F	130	D	~2.0	D	
6	VCFS29	F	132	D	~1.5	D	
7	VCFS33	M	115	ND	-	ND	
8	VCFS34	M	171	ND	-	ND	
9	VCFS36	M	180	D	~ 2.5	D	
10	VCFS38	M	161	ND	-	ND	ND
11	VCFS51	F	99	ND	-	ND	
12	VCFS63	F	207	ND	-	ND	
13	VCFS64	F	146	D	~2.1	D	D
14	VCFS73	M	164	D	~1.6	D	
15	VCFS123	F	139	ND	-	ND	ND
16	VCFS135	M	126	D	~2.0	D	D
17	VCFS136	M	128	ND	-	ND	ND
18	VCFS137	F	72	ND	-	ND	ND
19	VCFS138	M	96	D	~2.4	D	D

[1]ID, patient identification; [2]F, female and M, male; [3]D, deleted and ND, not deleted.

19 samples. The validation of a larger sample size by WES would be very costly.

A limitation of the developed TaqMan qPCR assay is that like FISH, it may give false negative results if the deletion is atypical, occurring elsewhere in the 22q11.2 region (Rauch et al., 2005; Stachon et al., 2007). This TaqMan qPCR assay detects the common 22q11.2 DS deletions implicated in 95% of affected patients. Clinically suspected cases testing negative need to be reassessed, which may warrant further molecular investigations. To the best of our knowledge, a duplex *HIRA*/*TES* TaqMan qPCR method has not been previously used for the molecular diagnosis of the 22q11.2 DS. TaqMan-based dosage analysis PCR, described here, is a rapid, sensitive, specific and low-cost molecular method to identify cases with common microdeletions in 22q11.2 DS.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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