Genotyping Sri Lankan women with polycystic ovary syndrome (PCOS): towards a novel screening tool

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(Index words: PCOS, single nucleotide polymorphism, allele specific qPCR)

Abstract

Introduction: Polycystic ovary syndrome (PCOS) is the multigenic, endocrine disorder of young women. Inheritance of PCOS is likely to be oligogenic and genetic basis remains largely unknown. Screening the candidate genes of PCOS and their SNPs individually is time consuming. Hence, developing a tool that would help in screening multiple candidate genes simultaneously is essential to determine the exact genetic basis of PCOS.

Objectives: This study aimed to develop a simple and cost-effective genetic screening tool to simultaneously genotype 16 single nucleotide polymorphisms (SNPs) of PCOS.

Methods: The genetic screening tool was developed using allele specific real time quantitative PCR (AS-qPCR) in 96 well PCR plate. Eight SNPs identified in our previous study as well as 8 SNPs identified from other reported studies that had a strong association in the etiology of PCOS were used to develop the tool. Samples from our previous study were reanalyzed using the developed genetic screening tool. Genetic screening tool results were validated with Sanger sequencing.

Results: Totally 10 AS-qPCR runs (160 reactions = 16SNPs*10runs) were performed using the developed tool and all except 3 genotype results agreed with Sanger sequencing. The tool showed 100% specificity and 96% sensitivity.

Conclusion: The developed genetic screening tool has excellent potential in determining the genotype of multiple SNPs of PCOS simultaneously. This tool is highly suitable for developing countries as a cost effective and accurate early genetic screening test for PCOS. Thus, provides a reliable, fast and user-friendly genotyping method facilitating a wider implication in clinical practice.

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uniform guidelines for its diagnosis and management among the adolescent population. Although the clear cause for PCOS remains unknown, both environmental and genetic factors have been implicated. The composite of evidence suggests that several mutations or polymorphisms in candidate genes involved in steroid, gonadotropin and insulin pathways closely interact to set the stage for the development of PCOS [9].

The community prevalence of PCOS is 6.3% among young Sri Lankan women [10] and is significantly high on an age specific basis when compared to other ethnic groups. PCOS has become a major health problem among women of reproductive age, with major metabolic implications that can cross generations.

Clinical expression of symptoms varies through the woman's life cycle and its expression in the reproductive period is noteworthy [11]. Hence, it is difficult to confirm the diagnosis of PCOS at any given phase of a woman's life. The lack of an accurate genetic diagnostic testing for PCOS is a problem that has arisen due to its heterogeneous clinical expression and phenotype. The genetic cause of the disease should be identified well before puberty. Identification of genetic risk factors which predispose to PCOS will facilitate early diagnosis, effective treatment and management of the disease from adolescence, thus reducing long term risks of early onset of type 2 diabetes mellitus, cardiovascular disease along with female infertility. However, involvement of multiple SNPs in PCOS is one of the major reasons for the delay in identifying the genetic cause of the disease. Hence, there is a need for the development of a tool that can screen multiple SNPs of PCOS simultaneously.

A study by Chen et al. (2010) identified SNP markers associated with PCOS using micro array chips and totally 45 SNPs can be genotyped by this method [12]. However, this method is not suitable for a developing country like Sri Lanka, since the cost of the probe and chip is very much high and cannot be affordable by the developing countries.

This study aimed to develop a genetic screening tool that can be affordable by the developing countries as well as involve simple techniques which can be performed locally in a reliable and cost effective manner to screen multiple candidate genes simultaneously to determine the exact genetic basis of PCOS at the early stage.

Methods

This work received approval from the Ethical Review Committee, Faculty of Medicine, University of Colombo, Sri Lanka (Protocol No EC-14-044). Consecutive women conforming to inclusion criteria were recruited from the Endocrine Clinic of the University Unit, Colombo, Sri Lanka. Diagnosis of PCOS was based on the Rotterdam criteria [13,14].

Sample size calculation

The Schlesselman case control study formula was used for sample size calculation [15]. Details of sample size calculation were described in our previous reports [16-18].

Recruitment of Subjects

Inclusion criteria: Inclusion criteria were women whose symptoms manifested from adolescent years (11-19 years WHO), with all 3 diagnostic criteria present from 16-19 years of age [19]. The lower limit of age selection was based on the mean age of menarche in Sri Lanka being 13 years and leaving an allowance of two additional years for regularization of menstruation [20].

Anovular PCOS or amenorrhea/oligomenorrhea: Anovular cycles are defined when the cycle length is more than 35 days, and the lack of demonstrable ovulation by mid cycle and luteal phase ultrasound scans, and mid-luteal serum progesterone [19]. Amenorrhea – absence of menstrual periods for six months or more in a woman who has previously been menstruating. Oligomenorrhea – menstrual periods occurring at intervals of greater than 35 days, with only four to nine periods in a year.

Polycystic ovaries on ultrasound: defined by trans-vaginal or trans-abdominal ultrasound scan of ovaries, performed within the first 5 days from the onset of menstruation, and finding 24 or more follicles, measuring between 2 and 9 mm and/or an ovarian volume >10 cm³ [13,14].

Hyperandrogenism: Clinical evidence of hirsutism by modified Ferriman-Gallwey score (mFG) ≥8, serum testosterone (T) >3.5 nmol/L [19].

Exclusion criteria: Exclusion criteria included inherited disorders of IR such as Rabson-Mendenhall syndrome, Cushing syndrome, hyperprolactinaemia, untreated primary hypothyroidism, congenital adrenal hyperplasia or an androgen secreting ovarian/adrenal tumor; those taking corticosteroid, antiepileptic or antipsychotic drugs, history of hormonal contraception within the previous 6 months, pregnancy and the first postpartum year.

Control sample: Concurrently asymptomatic, normo-androgenic, normal cycling since adolescence, non-medicated, consenting women of reproductive age in whom PCOS was objectively excluded by clinical, biochemical and ultrasound assessment, were recruited as controls. The control subjects were recruited from a single work setting where health promotion programs were conducted from 2012 (3 years before the study). Working women of similar ethnic and social background as the affected subjects were invited to participate in the study.

Development of genetic screening tool using plate based real time allele specific quantitative PCR

A genetic screening tool was developed to simultaneously determine the genotypes of 16 SNPs of PCOS by allele specific real time quantitative PCR (AS-qPCR)
using a 96 well PCR plate. Eight SNPs identified in our previous study [16] as well as 8 SNPs identified from the literature of other reported studies from Asia that had a strong association in the etiology of PCOS were selected to develop the method (Table 1).

Designing of primers for AS-qPCR was based on a modified version of CADMA (competitive amplification of differentially melting amplicons) [21]. This method uses 3 primers: two allele specific primers and one common primer. Of the 2 allele specific primers, one primer is designed to amplify only the mutated allele (mutant primer) and the other primer amplifies only the wild-type allele (wild type primer). The common primer was designed to amplify both wild type and mutant alleles. Details of primer designing are described in our previous study [16].

The AS-qPCR was carried out using qPCR-HRM mix (5X Solis BioDyne) and the allele specific primer for each SNP was added separately into the wells along with a common primer followed by the addition of templates (Figure 1a and 1b). The final reaction mix for each assay consisted of 1X qPCR-HRM mix allele specific primer (0.5 µM), common primer (0.5 µM), template (20 ng) and adjusted the volume up to 10 µL with deionized water.

The PCR cycling conditions for AS-qPCR was as follows: Initial denaturation 95°C for 15 minutes, followed by 40 cycles of denaturation (95°C for 15 seconds), annealing (60°C for 20 seconds) and extension (72°C for 20 seconds). Thereafter, high resolution melting (HRM) was performed from 50°C to 95°C with a temperature increase of 0.2°C/s with 50 acquisitions/°C.

Each single run consisted of a patient sample, positive control samples of respective SNPs and negative controls. Totally 10 randomly selected patient samples from our previous study were genotyped (totally 10 runs) using the screening tool. The genotype was determined by the Cq value of the amplification. To validate the plate based AS-qPCR method, the region flanking the polymorphic site was PCR amplified from genomic DNA using specific primers and then custom sequenced (Macrogen Inc, Korea) by Sanger sequencing method.

Table 1 depicted the 16 SNPs used for developing the screening assay. Primers used for the genetic screening tool are listed in Table 2.

**Determination of sensitivity and specificity of genetic screening assay**

The scientific reliability of the developed genetic tool is measured by the ‘sensitivity’ and ‘specificity’ of the test. The following formula was used to calculate the sensitivity and specificity [22].

\[
\text{Sensitivity} = \frac{\text{Number of true positives}}{\text{Number of true negatives} + \text{number of false negatives}}
\]

\[
\text{Specificity} = \frac{\text{Number of true negatives}}{\text{Number of true negatives} + \text{number of false positives}}
\]

| SNP of present study | SNP from other reported studies |
|----------------------|---------------------------------|
| FTO (rs9939609)      | AR (rs5919393) [26]             |
| FSHR (rs6165)        | INSR (rs2059807) [27, 28]      |
| FSHR (rs6166)        | THADA (rs13429458) [28]        |
| FSHB (rs6169)        | THADA (rs12478601) [28,29]     |
| INSR (rs1799817)     | FSHR (rs2349415) [30,31]       |
| GnRH (rs6185)        | FSHR (rs2268361) [30,31]       |
| LHB (rs1800447)      | SHBG (rs9913778) [32]          |
| LHCGR (rs2293275)    | SHBG (rs6259) [32, 33]         |

*FTO* – fat mass and obesity-associated gene; *FSHB* – follicle stimulating hormone beta subunit; *FSHR* – follicle-stimulating hormone receptor; *INSR* – insulin receptor. *GnRH* – gonadotropin-releasing hormone; *LHB* – luteinizing hormone beta subunit; *LHCGR* – luteinizing hormone/choriogonadotropin receptor. *AR* – androgen receptor; *THADA* – thyroid adenoma associated gene; *SHBG* – sex hormone-binding globulin.
Table 2. Primers of selected SNPs used for the development of genetic screening tool

| Gene | SNP | Primer Sequences (5'→3') |
|------|-----|---------------------------|
| **FTO** | rs9939609 | F. wild type (T) – TAG GCT CCT CGC GAC TGC TGT GAA TAT T  
F. mutant (A) – TAT GTT CAT TGC GAC TGC TGT GAA TAT A  
Comm reverse – GAG TAA CAG AGA CTA CTC AAC TGC ATC AC  
F. seq primer – CTG GCT CTT GAA TGA AAT CAG TGC TGT CGT C |
| **FSHB** | rs6169 | F. conn primer – GTA CCT TCA AGG AAC TGG TAT  
R. wild type (C) – CCG GCA CTC TCA CTG TCG CG  
R. mutant (T) – CAG GCA CTC TCA CTG TTA CA  
R. seq primer – GCA CAG TAC AAT CAG TGC TGT CGT C |
| **FSHR** | rs6165 | F. wild type (T) – CAG AGA GAA TCT CTG AACC CCT AGT  
F. mutant (C) – CAG AGA GGG TCT CTG AGC CCT AGC  
Comm reverse – ACC CCA TGA TAT CTT CAC ATG GGT TGA A  
F. seq primer – CTG GCT CTT GAA TGA AAT CAG TGC TGT CGT C |
| **FSHR** | rs6166 | F. wild type (T) – AGG GAC AAG TAT GTA AGT AGA ACC AT  
F. mutant (C) – AGG GAC AAG TAT GTG AGT GGA ACC AC  
Comm reverse: CTC TTC AGC TCC CAG AGT CAC CA  
F. seq primer – CCA ATT TAC TCT AAT AAA GGT ATG CCA |
| **INSR** | rs1799817 | F. conn primer – ATG TCC CAC CCC CAC TGG ACT CAC AAC  
R. wild type (C) – TCG GTC ATG AAG GGC TTC ACC TGC CAT GAC  
R. mutant (T) – TAA GTC ATG AAG GGC TTC ACC TGC CAT AAC  
R. seq primer – CTG TGT GTA CGT GCC GGA CGA GTG GAG G |
| **GnRH** | rs6185 | F. conn primer – TGG CTG GAG CAG CCT TCC ACT CA  
R. wild type (G) – CGC CTA GCT GGC CTT ATT CTA CTG AGC TG  
R. mutant (C) – CTA CTA GCT GCC CTT ATT CTA CTG ACA TC  
R. seq primer – CTG ACT CTG ACT TCC ATC TTC TGC TGC AGC G |
| **LHB** | rs1800447 | F. wild type (A) – ATT GCA TTG ATG GGG TGG CAA CA  
F. mutant (G) – ATG GCA TTG ATG GGG TGG CAG CG  
R. conn primer – ATC CAG GGA GCC GCT TGC GAC A  
F. seq primer – CTG CCT CTG TGG GCC GCA GCC GCC GCC G |
| **LHCGR** | rs2293275 | F. wild type (T) – GTA TGG AAA TAC TTA CAG TGT TTT GTG AT  
F. mutant (C) – AGC CGG CAA ATA CTT ACA ATG GTG TTT TGT GAC  
R. conn primer – CAA TGT GAA AGC ACA GTA AGG AAA ATG A  
F. seq primer – CAA TTG CAA AGA AAA AAT TCC CAT TTT A |
| **AR** | rs5919393 | F. wild type (C) – ACC ACT CAC CCT TTT TG CTA GAT AGT GCC  
F. mutant (T) – CTT ACT CAC CCT TTT TG GCC AGT AGC ACT  
Comm reverse – CTC CAA AGA GTC TGT CCA CAA TGA AAG T  
F. seq primer – CAG GGA CCA GTG TGA GAA TG |
| **INSR** | rs2059807 | F. conn primer – GCA TTT TAC TCA ACC TCA CTG CAT CAG CCT  
R. wild type (T) – GAA GTA TGT GAA TCA GAC CTC TTT CTG AT  
R. mutant (C) – TGG GTG TGT GAA TCA GAC CTC TTT TTC AC  
R. seq primer – CAA CGA CCC CAA ATC ACA G |
| **THADA** | rs13429458 | Comm forward – TGC TGT GCA AAG TTA GAA GAT GAA AC  
R. wild type (T) – GAA GCC AGG GTA TAG GTG TAT GTA ATC AGT CTT  
R. mutant (G) – TGG GCC AGG GTA TAG GTG TAT GTA ATC AGT CTG  
R. seq primer – ACT GAC ATG TTT CCC ATC T |

(Continued)
**THADA**  
rs12478601  
F. wild type (C) – TGG CAG TCC TGC TGG TCT TGG TTA GTA CGA C  
F. mutant (T) – GAA CAT TCC TGC TGG TCT TGG TTA GTA CAA T  
Comm reverse: GTA AAG CCC GGG TCC TAA CAT TTT ATT GA  
F. seq primer – TGG AGT TGG ACA GGT AAT AG

**FSHR**  
rs2349415  
F. comm primer – ATA AAA ACA GGT GTC AGC GAT TGG A  
R. wild type (A) – GAA CAG TGT CAC TGA ACT ACA GCC AAT TGA  
R. mutant (G) – TGG CAG TGT CAC TGA ACT ACA GCC AAT TGG  
R. seq primer – TCA ATC CTA CCC CCT GAC AG

**FSHR**  
rs2268361  
F. wild type (C) – ACC ATG CTG TGA GAC GAA GCC ATC TTC TC  
F. mutant (T) – CCT ATG CTG TGA GAC GAA GCC ATC TTC TT  
R. comm primer – AAT GAA AGA TCT CAA TCC CAG GCC ACT  
F. seq primer – CAG AAT GAT ACA GTG GGT AGA

**SHBG**  
rs9913778  
F. wild type (C) – TGG TCC ATC AGC TCT TCT TCC TCA  
F. mutant (T) – GAA TCC ATC AGC TCT TCT TCC ATT  
R. common primer – CTG ACA TGT CCC TAC TCA GC  
F. seq primer – CAG AAT AGA TCT CAA TCC CAG GCC ACT

**SHBG**  
rs6259  
F. wild type (G) – TGG TTT GCA CTA CCT CCC TCT AGG AGC AG  
F. mutant (A) – GAA TCT GCA CTA CCT CCC TCT AGG AGC AA  
R. common – CAT TCA GCC AAA AAG AGG TGG AAG AGT  
F. seq primer – AAG GGA GCC GGC ACA TTT T

F – forward; R – reverse; Comm – common; Seq – sequence

**Table 3. Genetic screening tool results of 10 randomly selected patient samples**

| SNPs         | Patients sample | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  |
|--------------|----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| FTO – T/A (rs9939609) | AA  | AT  | T T | AA  | AT  | T T | AT  | T T | T T | AA  |     |
| FSHR – G/A (rs6165)    | GG  | GG  | GA  | AA  | GA  | AA  | GG  | GA  | GG  | GA  |     |
| FSHR – G/A (rs6166)    | GG  | GG  | GA  | AA  | GA  | AA  | GG  | GA  | GG  | GA  |     |
| FSHB – C/T (rs6169)    | CC  | CC  | CC  | CC  | T T | T T | CC  | CC  | CC  | T T | CC  |
| INSR – C/T (rs1799817) | CC  | CC  | CC  | CC  | CC  | CC  | CC  | CC  | T T | CC  |     |
| GnRH – G/C (rs6185)    | GG  | GG  | GG  | GG  | GG  | GG  | GG  | GG  | GG  | GG  |     |
| LHB – T/C (rs1800447)  | CC  | CC  | CC  | CC  | CC  | CC  | CC  | CC  | CC  | CC  |     |
| LHCGR – A/G (rs2293275) | GA  | AA  | GA  | GA  | GA  | AA  | GG  | GA  | GG  | GA  |     |
| AR – C/T (rs5919393)   | T T | CC  | T T | CC  | CC  | CC  | T T | CC  | CC  | T T |     |
| INSR – A/G (rs2059807) | AA  | AA  | AG  | AG  | AA  | AA  | GG  | AA  | AA  | AA  |     |
| THADA – A/C (rs13429458) | AC  | AA  | AA  | AC  | CC* | AA  | AA  | AA  | AC  | CC* |     |
| THADA – C/T (rs12478601) | CC  | CC  | T T | T T | T T | CC  | TT  | CC  | CC  | CC  |     |
| FSHR – T/C (rs2349415) | TT  | TT  | TT  | CT  | TT  | TT  | TT  | CC  | CT  | T T |     |
| FSHR – C/T (rs2268361) | CC  | CC  | CT  | T T | CC  | CC  | TT  | CC  | CC  | CT  |     |
| SHBG – C/T (rs9913778) | CC  | CC  | CC  | TT* | CC  | CT  | CC  | CC  | CC  | CC  |     |
| SHBG – G/A (rs6259)    | GG  | GG  | GG  | GG  | GG  | GG  | GG  | GG  | GG  | GG  |     |

* Not agreed with the sequencing results
Results

The AS-qPCR screening tool was developed using a 96 well PCR plate. A patient sample was simultaneously genotyped for 16 SNPs in a single run. Table 3 depicts the results of 10 randomly selected patient samples genotyped for 16 SNPs using the screening tool.

The screening assay results of 10 randomly selected patient samples were validated with Sanger sequencing. Figure 2 shows the results of AS-qPCR and respective Cq values. The Cq values <30 indicates positive amplification of the target. If the Cq value of one primer set is > 30, it indicates the absence of the respective allele in the sample; thereby homozygous towards the other allele. When both primer sets show Cq value < 30, the sample is heterozygous.

With reference to the Table 3 results, sensitivity and specificity of the developed tool are calculated using following formula;

Sensitivity of the tool = \[ \frac{\text{Number of true positives (69)}}{\text{Number of true positives (69)} + \text{number of false negatives (3)}} \] * 100 = 96%

Specificity = \[ \frac{\text{Number of true negatives (91)}}{\text{Number of true negatives (91)} + \text{number of false positives (0)}} \] * 100 = 100%
The developed genetic tool showed 100% specificity and 96% sensitivity. Two patients’ samples genotyped for THADA (rs13429458) and one patient sample genotyped for SHBG (rs9913778) polymorphisms were detected as homozygous although they were heterozygous. There were no false positive genotype results.

Discussion

The development of biotechnology to be benchmarked as ‘state of the art’ in Sri Lanka is still to be achieved. Although many initiatives have been adopted in the field, there is much more to do for achieving a satisfactory level. A major gap is the lack of advanced technology in the form of instruments (e.g. Capillary electrophoresis for Sanger sequencing) in Government institutions. Therefore, most of the Sanger sequencing tests of PCR products as well as DNA sequencing for next generation sequencing (NGS) are outsourced to overseas and require transportation charges as well as tedious documentation procedures. Hence, there is a real need to develop a simple and cost-effective genotyping method which can be performed locally to match the growing demand for rapid and reliable genotyping of medical conditions that are commonly encountered, such as PCOS.

In recent years, conventional polymerase chain reaction (PCR) techniques have been replaced by quantitative real-time PCR (qPCR). The benefits of qPCR in relation to conventional PCR include speed, reproducibility and quantitative ability. Additional operational advantages of qPCR include greater sensitivity and reproducibility, with the potential to replace conventional PCR in routine diagnostic practice. The present study developed AS-qPCR in 96 well PCR plates for genotyping multiple SNPs of PCOS which work on the principle of high-resolution melting in real time PCR instruments. Since the real time PCR instruments are currently available in most Government institutions as well as in private laboratories, the developed method can be easily performed locally without the need for advanced molecular biology techniques. It is noteworthy, that the principles of HRM and AS-qPCR have been used and recommended by some lead centers for genotyping [21, 23-25].

The cost for Sanger sequencing (Macrogen Inc, Korea) for 250 samples is 1,750 USD (7 USD per read). As the Sanger sequencing facility is not available in many institutions in Sri Lanka, samples need to be transported overseas for genotyping. Hence, the turnaround time to obtain results is minimum 5 days after DNA extraction. However, the pursued AS-PCR method can be carried out locally and the turnaround time after extraction of DNA is maximum 3 - 4 hours. Nevertheless, the developed tool eliminates the need for overseas transportation thereby reducing the overall cost and the total cost for HRM/AS-PCR for 250 samples is around 150 USD. Although cost of Sanger sequencing is low in developed countries, it is still an expensive method for a developing country like Sri Lanka.

The AS-qPCR in 96 well PCR plates provide a convenient and cost-effective method for genotyping SNPs of common polymorphisms of PCOS. Major limitation of the tool is that initially samples need to be validated by sequencing to confirm the results. However, by applying samples of known genotypes as a reference, the assay can be used for reliable genotyping of samples of unknown status.

Before using a genetic test in clinical settings, it is important to be sure that the test is valid and efficient. The validity of a screening test is based on its accuracy in identifying diseased and non-diseased persons. Validity of the genetic tool is calculated by using sensitivity and specificity. It can only be determined if the accuracy of the screening test can be compared to with a “gold standard” method. Hence, in this study genetic tool results were validated by Sanger sequencing method, which is considered as “gold standard” for validating the sequence of specific genes.

Ideally, a test should provide a high sensitivity and specificity. The developed genetic tool had the 96% sensitivity and 100% specificity which means the tool has the ability to correctly identify the patients with mutated genes and normal genes. Thus, confirmed an ideal tool for screening multiple SNPs simultaneously. Therefore, the genetic tool can be used in the clinical settings after validation.

This tool is highly suitable for the developing countries, where no advanced techniques and instruments are available. Since real-time PCR instruments are standard equipment in most molecular diagnostic laboratories, the novel method developed can be easily performed, with potential to be rated as the most cost-effective and efficient genotyping technique. Utilizing samples of known genotypes as controls, the tool can be applied for clinical settings. Additionally, this method is more feasible to use in a country like Sri Lanka, where only a few SNP variations have yet been identified in the population.

More importantly, this tool can be modified to different populations based on their occurrence of SNPs and also can be used in various diseases that are associated with multiple candidate genes. We propose that this newly developed tool has potential to pave the path towards better understanding of the causative factors and those associated with the graded risks of complications of PCOS. Overall, such an approach may help foster a better understanding of the pathophysiology of PCOS in different subgroups and populations. Such knowledge could then be leveraged to devise the most optimal screening and effective management for young women with PCOS from different subgroups and ethnicities.
In conclusions, allele specific real time PCR are simple, fast, cost-effective and efficient genotyping techniques, feasible in many diagnostic units, as real time PCR instruments are standard equipment in most molecular diagnostic laboratories. Moreover, the developed novel genetic screening tool has excellent potential in determining the genotype of multiple SNPs of PCOS simultaneously. This tool is highly suitable for developing countries as a cost effective and accurate early genetic screening test for PCOS.

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Author contributions

The study was designed by CW, SW and NVC. Experiments were performed by UB and KM, and UB wrote the scripts and performed the statistical analysis. Writing, reviewing, and editing were done by UB, CW, SW and NVC.

Declaration

Ethics approval and consent to participate: This study was approved by the Ethics Review Committee (EC-14-044), Faculty of Medicine, University of Colombo, Sri Lanka. Written informed consent was obtained from all participants.

Data access: The data generated in this study have been submitted to the Figshare repository (https://figshare.com/articles/Complete_Gene_results_BMC_sav/11637264).

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Conflicts of interest: The authors declare that they have no competing interests.

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