Evaluation of an in house genetic testing method for confirmation of Prader - Willi and Angelman syndromes in Sri Lanka

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Abstract

Introduction Prader-Willi syndrome (PWS, MIM 17620) and Angelman syndrome (AS, MIM 105830) are caused by imprinting defects of chromosome 15q11-13, with loss of maternal gene expression causing AS and paternal gene expression causing PWS. The diagnosis, once established in most cases using a methylation sensitive PCR test, enables appropriate therapeutic interventions and avoids the need for further genetic investigations. Genetic testing for PWS/AS is limited in Sri Lanka (and other low and middle income countries) mainly because parents are unable to pay for testing as these are not funded by the health service. Methods Ninety cases (44 Male and 46 female) with clinical features suspicious of PWS (n= 37) and AS (n=53) referred by a paediatric endocrinologist and a paediatric neurologist were recruited. Clinical information and blood samples were obtained following informed consent. DNA was extracted and methylation sensitive PCR (MS-PCR) was performed following bisulfite modification of DNA using an in house method and a kit. Results were validated using known positive controls. Parent child trio DNA samples were used in cases with confirmed PWS and AS to determine if the disease was due to a deletion or uniparental disomy. The cost of the MS-PCR testing the two modification methods and the microsatellite analysis was determined. Results Among suspected PWS cases 19/37 were positive while 5/53 suspected AS cases were positive. The lower identification rate of AS is probably related to the overlap of clinical features of this condition with other disorders. The kit based modification method was more reliable, less time consuming and cost effective in our laboratory. Conclusions The kit based modification followed by MS-PCR described here enables more affordable genetic testing of suspected PWS/AS cases and this is likely to improve patient care by targeting appropriate therapy for affected cases. Parental genetic counselling is made possible regarding the low recurrence risk, especially where a deletion or uniparental disomy are confirmed. In MS-PCR negative cases with a strong clinical suspicion of AS, UBE3A mutation testing is required. In addition, imprinting centre mutation/ deletion testing may also be needed in strongly clinically suspected, MS-PCR negative PWS and AS cases.

Introduction

Prader-Willi syndrome (PWS, MIM 17620) and Angelman syndrome (AS, MIM 105830) are genetic diseases caused by an imprinting defect of chromosome 15q11-13 [1, 2, 3, 4]. The estimated prevalence of PWS is 1 in 10,000 to 25,000 live births [3] and of AS is between 1 in 10,000 to 12,000 live births [2, 5]. Severe hypotonia and feeding difficulties in early infancy followed by weight gain and excessive eating in later childhood characterize PWS. Developmental delay, cognitive impairment (mean IQ of 60), hypogonadism, characteristic facial features, strabismus, scoliosis, short stature, behaviour and psychiatric problems are associated with PWS. Morbid obesity and type 2 diabetes mellitus are sequelae of the polyphagia, unless strict dietary controls are started in early life [6]. Growth hormone therapy is recommended for selected PWS cases [7]. Angelman syndrome is characterized by severe developmental delay, limited speech, gait ataxia and tremulousness of the limbs associated with a happy demeanour that includes inappropriate laughter. Seizures are common and associated with a high voltage activity on

electroencephalography (EEG) [8]. In most cases, expressive speech is limited, and alternative, augmented communication is recommended and should be introduced early in affected cases [5]

The chromosome 15q11-13 is an imprinted region where only one parent's copy of the gene is expressed with the second parental copy becoming methylated to transcriptionally inactivate it. The imprinting process, regulated by an imprinting centre (The PWS-IC is a 4.1-kb region, which spans the *SNURF/SNRPN* promoter and exon 1 and the AS-IC is an 880-bp sequence located ~ 35 kb centromeric of the PWS-IC) occurs in early post zygotic life and is an epigenetic mechanism for genetic disease. [9]

Around 65 – 75% of PWS [1, 10] and 60-75% of AS patients [2] have deletions involving chromosome 15q11-13. The common deletions are approximately 6Mb (type 1: 40% cases) and 5.3Mb (type 2: 60% cases) [10, 11, 12]. In PWS, the deletion involves loss of paternally expressed genes while the hemizygous 15q region contains the methylated (transcriptionally inactive) maternal genes. In AS, the maternal 15q contains the deletion with the hemizygous 15q region containing methylated paternal genes. Around 20 – 30 % of PWS cases [1] and 2-5% of AS cases [2] have uniparental disomy (UPD) as the mechanism causing the disease: affected PWS cases inherit two copies of the imprinted maternal 15q region (maternal UPD) with no copies of the non-imprinted paternal genes while in affected AS cases, there are two copies of the imprinted paternal 15q region (paternal UPD) with absence of the non-imprinted maternal genes [11]. Imprinting centre anomalies (deletions, point mutations) account for around 1-3% of cases with PWS [1] and 2-5% of AS cases [2]. Around 10 % AS cases have mutations of the UBE3A gene located within 15q11.3 [2, 13].

PWS patients with UPD have higher verbal IQs, milder behaviour problems and a higher risk of psychosis and autistic spectrum disorders [3]. Among AS cases with UPD, better physical growth, psychomotor development, language ability, and fewer movement abnormalities and ataxia and a lower prevalence of seizures are observed than do those with other underlying molecular mechanisms such as deletion and imprinting centre defects [14]. Deletion cases have hypopigmentation related to loss of the P gene on chromosome 15 [15].

Genetic testing of clinically suspected cases is recommended as it enables management strategies to be targeted early for affected cases including strict dietary control and growth hormone therapy for PWS and alternative communication strategies for AS. The first line investigation to confirm the diagnosis of PWS or AS is the detection of the abnormal pattern of methylation of chromosome 15q11-13 [16, 17]. Methods used include methylation specific polymerase chain reaction (MS-PCR), which involves detection of the methylation pattern of the *SNRPN* gene [18], which is paternally expressed with the maternal copy being methylated. The investigation involves bisulfite treatment of DNA where the cytosine residues of unmethylated DNA is converted to uracil (the methylated allele is resistant to this change) followed by PCR using primers specific for the differentially methylated sites within the SNRPN region [18] Methylation specific multiple ligation dependent probe amplification (MS-MLPA) utilizes a one-step approach using multiple specific probes around the chromosome 15q region and methylation specific

restriction enzyme (Hha1) [19]. Droplet digital PCR involves bisulfite reduction followed by PCR utilizing a sensitive, digital capture methods [20, 21].

Both deletion and duplication cases have low sibling recurrence risks and confirmation of the disease and identification of its mechanism enables more accurate genetic counselling. Methods used to detect the deletion include fluorescence *in situ* hybridization (FISH) and chromosome microarray (CMA). Family studies using linked microsatellite repeats are able to differentiate between deletion and UPD and determine the parent of origin. Whole exome sequencing has identified 15q11-13 deletions as well as indicate presence of uniparental disomy [20], diagnose unsuspected autosomal recessive mutations of non-imprinted genes located in 15q11-13 as well as other, associated diseases. In rare cases, when PWS/AS is caused by a parental balanced chromosome translocation, karyotyping is required. In cases fulfilling the diagnostic criteria for PWS and AS, and are negative for the conventional methylation based investigations, imprinting centre anomalies (deletion, point mutation or methylation anomaly) will need to be investigated [20, 22]. Around 10% of patients with AS have a point mutation involving *UBE3A* and sequencing of this gene needs to be performed [13].

Sri Lanka is an Indian Ocean island with a population of nearly 22 million. It is a middle income country (per capita income $ US 4060-world bank 2019) with wide income disparity [23]. It has significantly reduced infant and childhood mortality and morbidity related to common, preventable infectious disease and genetic diseases are now an important contributor to these indices. Sri Lanka has a health service that is free at the point of delivery but genetic testing is of limited availability and is mostly paid for by the parents of affected children. The availability of low cost testing is essential to enable access to genetic testing as currently, most diagnoses are based on clinical criteria alone.

We report the development of a methylation specific PCR [18] and microsatellite assay to identify affected cases of PWS and AS among Sri Lankan patients. These finding are of relevance to other low and middle income countries attempting to deliver genetic diagnostic services.

Methodology

Cases (n=90, 46 female) were recruited based on clinical suspicion of a paediatric neurologist, and paediatric endocrinologists and referred to a clinical geneticist. Patients with at least two major and one minor characteristic of PWS [21] or at least 3 characteristics of AS by age 3 years [24] were recruited at the Lady Ridgway hospital, Colombo, following informed consent from the parent/care giver. Ethical approval for the study was obtained from the ethics review board of the Lady Ridgeway hospital, Colombo. Among this group were 37 cases suspected of having PWS and 53 suspected AS cases. A group of Sri Lankan cases who have already had methylation testing using methylation sensitive PCR for PWS/AS in an accredited UK laboratory were used for validating results obtained from this study.

Methylation specific PCR

DNA was extracted from blood of patients using the QIAamp DNA Mini kit and subjected to 1-hour incubation with sodium bisulfite followed by purification, desulfonation, neutralization and precipitation [25]. The treated DNA was used for PCR using methylation sensitive primers to detect the modified and unmodified alleles [18]. The PCR primers were designed to detect the SNRPN alleles namely the methylated maternal, unmethylated paternal and control primers for unmodified DNA. PCR amplification was carried out in a final volume of 25μl containing 50ng of template DNA, 0.2mM dNTPs, 1.5mM $MgCl_2$, 0.2μM forward and reverse primers, 1x PCR buffer (Tris HCl pH 8.3, 50mM KCl) and 1U of *Taq* polymerase (UC Biotech). The following thermal cycling conditions were used: initial denaturation at 94°C for 5min, 35 cycles of 94°C for 30s, 62°C for 30s, and 72°C for 30s with final extension at 72°C for 10 mins.

Due to the failure to obtain consistent and reproducible results by the above method the bisulfite conversion process was carried out using a kit [MethylEdge Bisulfite Conversion system (Promega)] which involved using a pre-mix to incubate the DNA for 16 hours for the bisulfite modification, and this was followed by PCR using the primers and conditions detailed above.

Microsatellite Analysis.

Only three families were available for microsatellite analysis using parent and child trios.

Six microsatellite markers D159646, D159817, D1581513, D159822, D159659 and FES on chromosome 15 were used based on previously described methods [26]. D159646, D159817, D1581513, D159822 are located within the deleted region while D159659 and FES are telomeric to the deleted region. The PCR reaction was carried using an initial denaturation at 94°C for 5min followed by 35 cycles of 94°C for 30s, 62°C for 30s, and 72°C for 30s with a final extension at 72°C for 10 min. PCR amplification was carried out in a final volume of 25μl containing 50ng of template DNA, 0.2mM dNTPs, 1.5mM $MgCl_2$, 0.2μM forward and reverse primers, 1x PCR buffer (Tris HCl pH 8.3, 50mM KCl) and 1U of *Taq* polymerase (UC Biotech). PCR conditions (concentration of primers, $MgCl_2$) were optimized, including the annealing temperature using gradient PCR.

Cost determination

Data was recorded regarding the costs of consumables for each sample to generate a result including labour costs.

Results

In-house bisulfite modification method

This was initially performed on 18 samples (AS-8, PWS-10). Due to a poor DNA yield following modification, PCR was performed as singleplex for maternal and paternal primers. Due to inconsistency of the in house modification method, the incubation time was doubled (to 2 hours) with no improvement and therefore increased to 16 hours. This too however was not successful.

Bisulfite modification using a kit

MS-PCR results confirmed the diagnosis in 19/37 (51.4 %) suspected PWS cases. In these positive cases, PCR results in the amplification of only the methylated maternal 174bp product and lacked the unmethylated paternal 100bp fragment. Among the suspected AS patients, there were 5/53 (9.4 %) confirmed cases of AS with the PCR products showing only the methylated paternal 100bp fragment and the absence of the unmethylated maternal 174bp band (Figure 1).

Total cost (consumables, chemical and reagents) for the MS-PCR using an in house bisulfite modification is Rs. 5000 ($ 27) (inclusive of repetitive test due to inconsistent results) while total cost for in house MS-PCR using a kit for bisulfite modification is Rs. 2600 ($ 14). The current cost of PWS/ AS testing using methylation specific MLPA in the private sector laboratories is Rs.12, 000 ($ 62).

Microsatellite Analysis.

Microsatellite analysis was carried out for 3 families and identified one case with a deletion and two cases with UPD.

Total cost (consumables, chemicals and reagents) for microsatellite analysis is Rs. 2,670 ($15). While no investigations are available currently in the private sector for determining UPD, a micro deletion screen using MLPA can detect the common 15q11-13 deletion and this costs around Rs.16, 010 ($ 88).

Discussion

At present, the diagnosis and management of PWS and AS in Sri Lanka mainly involves clinical suspicion only, as most parents are unable to pay for the cost of genetic testing. This study recruited children referred with a clinical suspicion of PWS/AS. The clinical diagnosis of PWS is established in the presence of at least 3 major and 2 minor criteria for children less than 3 years and 4 major and 4 minor criteria for children more than 3 years of age [21]. The AS diagnostic criteria list 5 features [24]. Recruitment for this study used a lower threshold of criteria for both PWS and AS with patients with 2 major and 2 minor criteria of PWS and at least 3 AS criteria. This was to reflect the needs of clinicians who wish to exclude the diagnosis in addition to confirming cases with a high probability of being affected. Among PWS suspected patients, there were 19/40 (48%) found to be positive on testing.

Among AS suspected cases, the diagnostic yield was lower (4/50; 8%) which is consistent with the overlap in clinical features between AS and other disorders associated with severe developmental delay, seizures and ataxia. Unfortunately, *UBE3A* mutation testing was not possible (due to non-availability of funds) in the test negative cases and therefore, the diagnosis of AS was not completely excluded in these cases.

In cases in whom there is a continued clinical suspicion of PWS or AS, methylation centre mutation or deletion has to be considered as this will be missed in MS-PCR testing. Maternally inherited *UBE3A* mutations will also not yield a positive result on MS-PCR but will cause AS in the patient.

A negative test is also useful for clinicians as this will guide them regarding the need to consider further investigations for establishing an alternative diagnosis. A positive diagnosis of PWS will justify very tight dietary regulation, monitor blood lipid and glucose levels, identify and manage the behavior problems and consider the option of growth hormone therapy. A confirmed diagnosis of AS will justify introduction of alternative augmented communication methods as expressive speech development is likely to be minimal. This can complement ongoing medical care of affected patients. In both cases, recurrence risks for a future pregnancy can be discussed especially after microsatellite analysis. In both conditions, confirmation of UPD or deletion as the cause of PWS or AS confers a low recurrence risk for the next pregnancy.

As Sri Lanka has significantly reduced common infectious causes of mortality in infancy and childhood, genetic disease has become more important in the cause of infant and childhood mortality and morbidity and establishing genetic aetiology is becoming more important. At present in Sri Lanka, the cost of genetic testing and its limited availability have prevented their widespread use for confirmation of PWS and AS.

The MS- PCR method using a kit based bisulfite modification in our laboratory generated reproducible results and is a cost effective and affordable test for cases with suspected PWS and AS features prior to more expensive investigations such as chromosome microarray or whole exome sequencing.

Our experience suggests that other low and middle income countries will also be able to offer such testing at an affordable cost to enable more accurate diagnosis of these conditions and thus improve patient care.

Conclusion

Methylation specific PCR was successful in diagnosing PWS and AS cases and is useful to confirm or refute the diagnosis of suspected cases. The in house MS-PCR using a bisulfite modification kit is less expensive than the in house modification method because the latter required multiple repetition to generate results. The in house MS- PCR performed in our laboratory using bisulfite modification by a kit is reliable and less expensive compared to currently available tests in Sri Lanka while the in house MS- PCR using in house bisulfite modification was discontinued due to inconsistence results. The MS-PCR negative, suspected AS cases merit clinical review to determine the need for further genetic testing prior to exclusion of AS while a negative result probably excludes PWS, unless very strong clinical suspicion supports the need for methylation centre mutation/ deletion testing.

Linkage studies would help families seeking reassurance prior to planning a further pregnancy but is not mandatory. Identification of the mechanism of PWS/AS is useful as both deletions and UPD are associated with low recurrence risk for further pregnancies and helps genetic counselling.

Abbreviations

AS – Angelman Syndrome

CMA- Chromosome Microarray

dNTPs – Deoxynucleotide triphosphates

DNA – Deoxyribo nucleic acid

EEG – Electroencephalography

FISH - Fluorescence in situ hybridization

HCl – Hydrochloric acid

IC – Imprinting center

IQ - Intelligence quotient

KCl - Potassium chloride

$MgCl_2$ - Magnesium chloride

MS- MLPA – Methylation Specific multiplex ligation-dependent probe amplification

MS- PCR – Methylation specific polymerase chain reaction

PCR - Polymerase chain reaction

PWS – Prader- Willi syndrome

UPD – Uniparental disomy

Declarations

Ethical Approval

Ethical clearance was obtained from the ethical committee of Lady Ridgeway hospital, Colombo (LRH/DA/03/2012)

Committee members.

Dr. (Mrs.) M.B.A.L.P. Wijesooriya, Consultant Paediatrician.

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Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests.

Author's contribution

Nirosha P: Conceptualization, Validation and analysis, Investigation, Resources (patient identification/ obtaining ethics approval/ obtaining informed consent), Data curation, Writing review and editing, Visualisation, Project administration, Funding acquisition. **Deepthi De Silva**: Conceptualization, Methodology, Validation and analysis, Resources (patient identification/ obtaining ethics approval/ obtaining informed consent), Writing review and editing, Visualisation, Supervision, Project administration, Funding acquisition. **Chandrasekharan NV**: Conceptualization, Methodology, Validation and analysis, Resources (purchase of chemical), Writing review and editing, Visualisation, Supervision, Funding acquisition. **Rathnayake P**: Conceptualization, Resources (patient identification/ obtaining ethics approval/ obtaining informed consent), Writing review and editing. **Atapattu N**: Conceptualization, Writing review and editing

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Figures
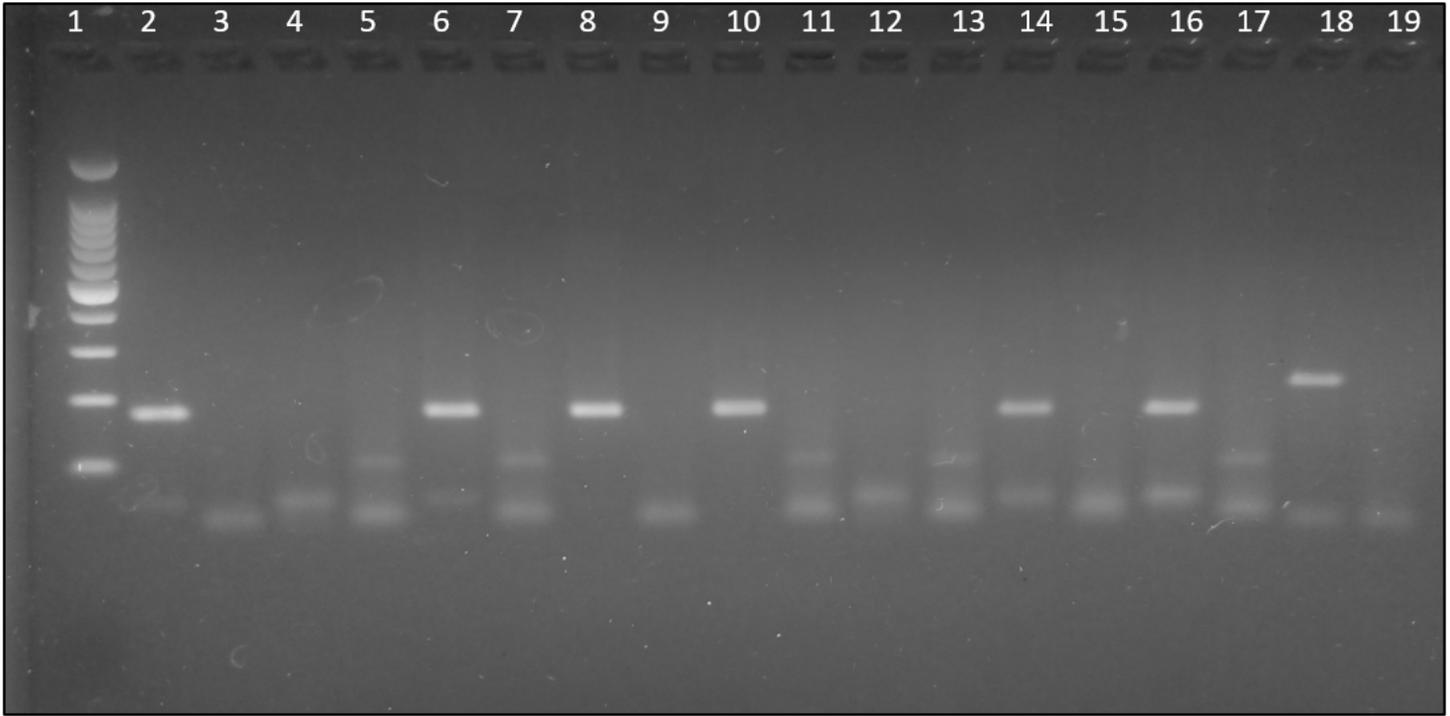
Figure 1

Methylation specific PCR analysis of patient samples (Kit method). Lane1: 100bp Ladder, Lane 2: PWS positive control (maternal primers), Lane 3: PWS positive control (paternal primers), Lane 4: AS positive control (maternal primers), Lane5: AS positive control (paternal primers), Lane 6: PWS patient 1 (Maternal primers), Lane 7: PWS patient 1 (Paternal primers) [PWS Negative] , Lane 8: PWS patient 2 (Maternal primers), Lane 9- PWS patient 2 (Paternal primers) [PWS Positive], Lane 10: AS patient 1 (Maternal primers), Lane 11- AS patient 2 (Paternal primers) [AS Negative], Lane 12: AS patient 2 (Maternal primers), Lane 13: AS patient 2 (Paternal primers) [AS Positive], Lane 14: PWS patient 3 (Maternal primers), Lane 15: PWS patient 3 (Paternal primers) [PWS Positive], Lane 16: control (Maternal primers), Lane 17:control (Paternal primers), Lane 18- Untreated genomic DNA, Lane 19- No template control.
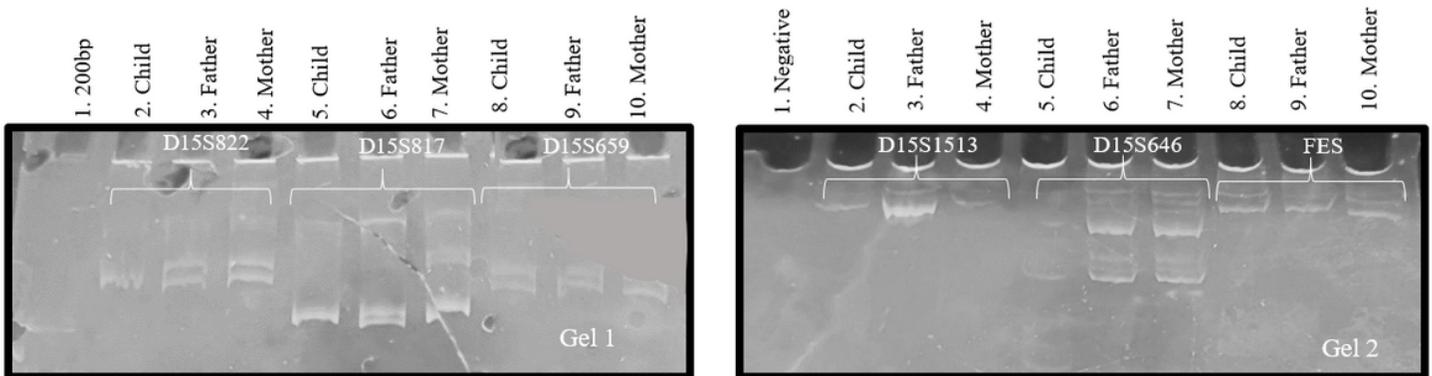
Figure 2

Microsatellite analysis of patient sample. Gel 1: Lane 1- 200 bp marker, Lane 2, 3, 4- D15S822 (Within the deleted Region), Lane 5, 6, 7- D15S817 (Within the deleted Region), Lane 8, 9, 10- D15S659 (telomeric to deleted Region). Gel 2: Lane 1- Negative, Lane 2, 3, 4- D15S1513 (Within the deleted Region), Lane 5, 6, 7- D15S646 (Within the deleted Region), Lane 8, 9, 10- FES (telomeric deleted region). [Paternal Deletion]