Evaluation of Clinical Features and Identification of 22q11 deletion among selected patients with cleft palate

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ABSTRACT

Cleft palate is the commonest multifactorial genetic disorder with a prevalence of 0.43-2.45 per 1000. It is associated with more than 400 syndromes. The objectives of this study were to evaluate the demographic background, clinical features and identify the 22q11.2 deletion in patients with cleft palate in Sri Lanka.

Cleft patients attending to the Regional Cleft Centre and Maxilo-facial Department, Teaching Hospital Karapitiya were recruited for this study. The relevant data were obtained from review of case notes, interviews and examination of patients according to a standard evaluation sheet. Quantitative multiplex polymerase chain reaction (PCR) was performed to identify the 22q11.2 deletion. A gel documentation system (Bio-Doc) was used to quantify the PCR product following electrophoresis on 0.8% agarose gel.

There were 162 cleft palate patients of whom 59% were females. A total of 92 cleft palate subjects (56.2%) had other associated clinical abnormalities. Dysmorphic features (25.27%) and developmental delays (25.27%) were the commonest medical problems encountered. The cleft was limited to the soft palate in 125 patients, while in 25 patients it involved both the hard and the soft palate. There were seven subjects with bifid uvula and five subjects with submucous cleft palate. None of the patients had 22q11.2 deletion in this study population. A multicentre large population-based study is needed to confirm the results of this study and to develop guidelines on the appropriate use of 22q11.2 deletion testing, which are valid for cleft palate patients in Sri Lanka.

Keywords: 22q11 deletion syndrome, cleft palate, congenital anomalies, polymerase chain reaction, Sri Lanka

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Introduction

Cleft palate is a congenital fissure in the roof of the mouth that results from incomplete fusion of the palate during embryonic development (1). It is the most common congenital malformation of the head and neck region. It is often associated with cleft lip and various other congenital anomalies (2). It contributes substantially to long-term disability in children, as well as tremendous emotional and financial stress for the affected individuals and their families . The treatment is a long-term process that starts soon after the birth and continues into the end of the second decade of life with multiple surgeries and long-term speech, orthodontics, audiological, medical, and dental care.

Development of the palate occurs between the 6th and 11th weeks of intrauterine life. Abnormalities of any of the critical events of development due to environmental, local or genetic predisposition result in clefts of the palate.[1-3]There are several causative factors that have been recognized in the aetiology of cleft palate. Disruption of the palatal mesenchyme which involves in palatal epithelial cell proliferation causes failure in palatal shelves' adhesion and fusion. This is the commonest factor associated with cleft palate. Abnormalities in palatal shelf movements with the growth of the tongue, can lead to widening of the gap between the two palatine

shelves and failure of meeting in the midline. Cleft palate that is seen in Pierre Robin sequence is an example for this type of failure (1-3). Failure of medial edge epithelial cell death can also lead to cleft palate. Medial edge epithelial cell death is an important event in the initiation of fusion of embryonic structures in the palatine shelves. Possible post-fusion rupture of the already fused membrane and failure of mesenchymal consolidation and differentiation of the palatine shelves may also contributed to the formation of cleft palate (1-3).

Prevalence of cleft lip and palate

The prevalence of the cleft palate with or without cleft lip varies according to a number of factors. The overall incidence of cleft palate with or without cleft lip is 1 in 1000 live births (4). Generally, the incidence of isolated cleft palate (without cleft lip) is 1 in 2000 live births. Submucous cleft palate is more common, with an incidence of 1 in 1200-2000 live births. The bifid uvula often occurs in isolation, without clefting of the palatal muscles (5).

There are variations in the prevalence rates of cleft lip and palate in different regions. Low birth prevalence of clefts (0.24 per 1000 live births) was found in Zambia (2). The prevalence rates of cleft palate were reported to be 0.43 and 0.48 per 1000 live births in Australia and California, respectively (7,8). The incidence of cleft lip and palate in Sri Lanka is 0.83 per 1000 live births, and the incidence of isolated cleft palate is 0.19 per 1000 live births. A positive family history has been found in 9.1% of cleft palate subjects in Sri Lanka (9). The incidence of cleft lip and palate has doubled during the last 50 years and tripled during the last 100 years (10). A 30-year follow-up study showed a clear trend of rapid increase in cleft lip and palate in Finland (11).

Etiology of cleft lip and palate

The etiology of cleft lip and palate is multifactorial. Several genetic and environmental factors interact with the process of morphogenesis of the primary and secondary palates (9). Isolated cleft lip and palate unaccompanied by any other malformation is an autosomal dominant inherited disorder, and the genes were found to be located on the short arm of chromosome 6. Other pedigrees show autosomal recessive and X-linked recessive patterns (12).

Trisomy 13, trisomy 18, velocardiofacial (VCF) syndrome, Pierre-Robin syndrome, fetal valproate syndrome, and oto-palato-digital syndrome are few of the syndromes that are associated with cleft palate (2). There are over 400 syndromes which include cleft lip and/or cleft palate as a component and are listed in the London Dysmorphology Database (2).

22q11 deletion syndrome

The chromosome 22q11 deletion syndrome (Mendelian inheritance in man database number 188400) is a relatively common genetic disorder characterized by congenital cardiac defects, cleft palate, velopharyngeal insufficiency, distinct facial features, immunological problems, learning disabilities, and psychological disorders (5,13,14). This syndrome is caused by deletion of chromosomal material from the long arm of chromosome 22 (22q), which leads to a wide but variable spectrum of effects.

The term velocardiofacial syndrome was used for the milder end of this deletion syndrome. These patients usually manifest palatal anomalies, distinct facial features, and learning disabilities (15). This disorder appears to occur as a result of failure or abnormalities in the formation of the 3rd and 4th branchial arch structures from which the affected organs and structures are derived.

22q11 deletion syndrome is one of the common syndromes associated with cleft palate. The prevalence of this syndrome has been estimated to be between 1 in 3800 and 1 in 6500 live births (13,16). Among infants born with conotruncal heart defects, 5% have been found to have a deletion of chromosome 22q11.2.[16] Approximately 5-8% infants with cleft palate had a 22q11.2 deletion (15). The prevalence of this deletion syndrome in Sri Lanka is unknown.

The 22q11.2 region is a hotspot for rearrangements due to deletions, duplications, and translocations. These rearrangements result in altered gene dosage (17-21). The most commonly deleted region of chromosome 22q11.2 involves the loss of a 3 Mb region in approximately 85% of cases. However a smaller nested deletion of 1.5 Mb is also described in

a further 10% of cases (14). The characteristic disease phenotype is caused by a haploinsufficiency of a series of 24-30 genes within the 22q11critical region (14).

This deletion occurs in about 94% of cases as a de novo event without preceding family history of a similar deletion. In about 6% of cases, the deletion is inherited from a parent (14).

Diagnosis of 22q11 deletion syndrome is mainly based on clinical evaluation and confirmed by genetic investigations. Early detection of 22q11 deletion is far more important as potential complications related to this syndrome can be identified early so that the condition can be managed prior to cleft palate repair. It is necessary to investigate these patients genetically for post-test genetic counseling (22).

Objectives of the study

The objectives of this study was to describe the socio demographic background, clinical features and the prevalence of the 22q11 deletion among patients with cleft palate who presented to a selected cleft palate center in Sri Lanka.

Materials and methods

Patients with isolated cleft palate (without cleft lip) were selected for the study. Patients were identified among those who were currently under review in the Regional Cleft Centre & Maxillofacial Department, Teaching Hospital, Karapitiya, Galle. All patients with isolated cleft palate registered in the clinic from 1 January 2001 to 31 December 2009 were included in the study. A total of 162 cleft palate patients participated in this study. Before enrolling in the study, the entire procedure of the research was briefly explained to the patients and in the case of children under 16 years old, to the parents or guardian. Steps had been taken to maintain the confidentiality of data. Before the evaluation of the patients, a written consent was obtained from all the patients and in case of the children, from parents or guardian.

The patients who consented to participate in the study were interviewed individually in detail by the researcher and data were recorded in an internationally accepted standard structured questionnaire. Complete evaluation of the patient was carried out including relevant history and full clinical examination. All the clinical notes and diagnosis cards were reviewed. Where necessary, patients were referred to special investigation units for procedures such as ultrasound scan, echocardiogram, computed tomography (CT) scan, magnetic resonance imaging (MRI) scan, hearing and visual investigations, etc. Feeding in infants and speech in older children and adults were evaluated by a qualified speech therapist.

All the patients with cleft palate who consented were included in the assessment of 22q11.2 deletion. One to three milliliters of venous blood was obtained from each patient for the molecular genetic analysis.

The antero-posterior and lateral views of face, anterior and posterior aspects of hands and feet of the subjects were photographed and securely stored in a computer. All photographs were reviewed according to the guidelines formulated by Tobias et al., (1999) by a clinical geneticist who was blind to the results of the molecular data. Physical growth of the subjects below five years of age were analysed by using a computer software WHO Anthro version 3.2.2 (January 2011) designed and recommended by World Health Organization (WHO) for nutritional surveys.

Ethical clearance was granted for the study by the Faculty of Graduate Studies, University of Keleniya, Sri Lanka.

Quantitative multiplex PCR

DNA was extracted by using commercially available human genomic DNA extraction kit (QIAamp DNA Mini Kit; Qiagen, Germany). Two sets of 300µl of whole blood from each patient were used to extract DNA. All the extracted DNA samples were quantified by using UV spectrophotometer [Thermo Spectronic-Genesys (TM)].

Ten sets of forward and reverse primers were selected for the semi-quantitative multiplex PCR in order to identify the 22q11.2 deletion from among the 40 primer sets described previously (23). Initially, eight sets of primers were selected from established sequence tag sites (STS) between the proximal and distal break points of the typically deleted region. Two other sets of primers were

selected, one outside the deleted region and the other specific for cystic fibrosis transmembrane conductance regulator gene (CFTR) on chromosome 7 to normalize small variations in DNA concentration and amplification efficiencies. All the selected primers were analysed by using the Basic Local Alignment Search Tool (BLAST) for non-specific alignments. All the selected primers were tested individually by PCR and the PCR conditions were optimized. The best combination of primers for the multiplex PCR was obtained using sequence tag site (STS) D22S609 (D1) with a fragment size of 300bp and the CFTR gene STS, SHGC35613 (S2) with a fragment size of 200bp. This was based on the ability to amplify both regions using the same PCR conditions as well as optimal separation of the PCR fragments during gel electrophoresis. Therefore it was decided to use these two primer sets for identifying the 22q11.2 deletion. PCR was carried out in a volume of 25µl using a thermal DNA cycler (Eppendorf, Germany). Human genomic DNA (100ng) from patients (P) and from a normal subject (N) were amplified using specific primer sets representing established STS markers spanning the 3 Mb TDR. For each PCR, an internal control of cystic fibrosis gene (SHGC35613) was also included. The annealing temperature for each primer set and the PCR conditions were optimized as described by Rolfs *et al* (24). Quantification of PCR products was carried out in the log phase (26 cycles of PCR) after electrophoresis using a gel documentation system (Bio-Doc). All dosage estimations were carried out using three independent PCR reactions. A ratio of 1N:1P indicated that there was no deletion, while a ratio of 2N:1P indicated a deletion.

Statistical analysis

Proportions of the same groups such as socio-economic status and growth parameters were compared using one way chi square test. Numerical data were compared using student T test. Epi Info TM 7.1.0.6 software was used for the statistical analysis.

Results

Gender, age ethnicity and geographic distribution

There were 323 patients with cleft palate without cleft lip, who attended the Regional Cleft Centre & Maxillo-Facial Department, Teaching Hospital Karapitiya over the period starting from 1 January 2001 to 31 December 2009. There were 187 females (57.9%) and 136 males (42.1%). Responding to the request to attend the routine clinic review, 162 patients attended the clinic and volunteered to participate in the study (50.14%). There were 95 (58.64%) females and 67 (41.36%) males.

The male to female ratio in the study sample was 0.71. According to the department of census and statistics in Sri Lanka the sex ratio in the southern province of Sri Lanka is 0.96 (Population and housing censuses in Sri Lanka, 2001).

Table 1: Comparison of sex ratio of the study sample and national figures (39)

Population	National figures (Southern province)*	Study sample	p value
Sex ratio	0.96	0.71	0.0148

(*Population and housing censuses in Sri Lanka 2001)

There was a significant different (p = 0.0148) in sex ratio of the study sample and the national figure for the southern province.

The age range was from 2 weeks to 49 years. There were 24 patients with less than or equal to 1 year of age. Most of the patients were pre-school children less than 5 years of age (51.23%).

Most of the subjects were from the Southern Province (90.12%) and majority of them were residing in the Galle district.

Table 2: Comparison of the ethnic composition with that of the Southern province of Sri Lanka (39)

Groups	Ethnic composition of the study sample**	Ethnic composition of the Southern province of Sri Lanka*	p value
Sinhalese	93.9%	95.2%	0.09
Moor	4.7%	2.5%	0.007
Tamil	1.4%	1.8%	0.5
Other	0.0%	0.5%	

(**Expressed as a percentage of the total number of subjects, n=148, excluding subjects from Western and Sabaragamuwa provinces). (*Population and housing censuses in Sri Lanka, 2001).

There were eighty four (n=84; 51.85%) first rank births, fifty (n=50; 30.86%) second rank births, twenty (n=20; 12.35%) third rank births, six (n=6; 3.7%) fourth rank births and two (n=2; 1.23%) fifth rank births among study subjects.

Table 3: Comparison of birth rank with the general population (40)

Birth rank	Percentage in the Sri Lankan population*	Percentage in study sample**	P value
1st rank	43.19%	51.85%	0.0001
2nd rank	34.79%	30.86%	0.07
3rd rank	15.73%	12.35%	0.03
4th rank	4.35%	3.7%	0.4
5th rank	1.29%	1.23%	0.8

(*Population and housing statistics, 2000)

(**Expressed as a percentage of the total number of subjects, n=162)

There was a statistically significantly higher incidence (p = 0.0001) of cleft palate among first rank births in the southern part of Sri Lanka (Table 3).

Variation of birth months

Twenty three (n=23; 14.20%) and nineteen (n=19; 11.73%) births in the study population were in the months of March and February. There were five (n=5; 3.10%) and seven (n=7; 4.32%) births in the months of October and September respectively.

Table 4: Comparison of monthly variation of births of cleft palate cases with data from the general population (41)

Month	Sri Lankan population* (percentage of births per month)	Current study**	p value
January	7.8%	7.4%	0.98
February	7.75%	11.73%	0.08
March	8.14%	14.2%	0.008
April	7.7%	8.02%	0.99
May	7.92%	7.41%	0.92
June	8.74%	6.79%	0.46
July	8.67%	9.88%	0.68
August	8.29%	9.26%	0.76
September	8.57%	4.32%	0.07
October	8.92%	3.09%	0.01
November	8.77%	8.64%	0.93
December	8.71%	9.26%	0.91

(*Extracted from population and housing statistics, 2010)
(**Expressed as a percentage of the total number of subjects, n=162)

A Significantly higher number of cleft palate cases in this study were born in the month of March ($p = 0.008$) when compared with the national birth incidence while significantly lower numbers were born in the month of October ($p = 0.01$).

Socio-economic status

Total monthly incomes of the families of the study sample were analysed according to the earnings from both parents and other monthly income of the family. The mean monthly household income in the southern province of Sri Lanka was Rs. 32,514 in 2009/10 (Department of Census and Statistics in Sri Lanka, 2009/2010). Monthly household income was categorized in to three categories. One hundred and two (n=102; 62.96%) subjects of the study sample belonged to the category of low income (< Rs. 20,000/- per month) families. Forty eight (n=48; 29.63%) subjects of the study sample belonged to the category of middle income (Rs. 20,000/- to 40,000/- per month) families.

Only twelve subjects (n=12; 7.4%) of the study sample belonged to the category of high income (> Rs. 40,000/- per month) families.

Birth history

Out of one hundred and sixty two (n=162) subjects, one hundred and fifty two (n=152; 93.8%) were born at term. Only ten (n=10; 7.2%) subjects were born preterm (before 36 weeks of gestation).

One hundred and four subjects (n=104; 61.2%) had a normal birth weight of 2.5kg or more and fifty eight subjects (n=58; 38.8%) had a birth weight less than 2.5kg. Two (n=2; 1.23%) subjects had birth weight below 1.5kg and ten (n=10; 6.17%) subjects had birth weight of more than 3.5kg.

Table 5: Comparison of low birth weight among the general population and study sample (The Sri Lanka demographic and health survey (SLDHS), 2006/07) (42-43).

Population	Study sample*	Sri Lanka	p value
Percentage of low birth weight	38.8%	17.3%	P < 0.0001

(*Expressed as a percentage of the total number of subjects, n=162)

There was a statistically significant ($p < 0.0001$) number of low birth weights among cleft palate subjects (Table 5).

Table 6 : Comparison of mean birth weight among the general populatio n and study sample (44)

Study	Present study	Nanayakkara *et al., 2011*	p value
Mean birth weight	2.73kg	2.85kg	0.003

There was a statistically significant difference ($p < 0.05$) in mean birth weight (Table 5.9) of the study sample and national figures (Nanayakkara *et al.* 2011).

Family history of congenital anomalies

There were forty two (n=42; 25.93%) subjects with a positive family history of congenital anomalies. The malformations included thirty cases (n=30; 18.52%) with cleft lip/cleft palate, seven cases (n=7; 4.32%) with a congenital heart disease, one (n=1; 0.62%) with neonatal death with unidentified malformations in their family history. Other minor congenital anomalies (limb anomalies, renal anomalies, gastro intestinal defects) were seen among eight (n=8; 4.94%) family members of study subjects. Thirty eight (n=38) family members of the study subjects had only isolated congenital anomaly and four (n=4) family members of the study subjects had a combination of anomalies.

Antenatal history

Information was collected regarding the antenatal history of the affected cases. One hundred and thirty two (n=132; 81.48%) had an uneventful gestational period. Thirty (n=30; 18.52%) mothers reported complications during their pregnancy. Twenty two (n=22; 13.5%) had various illnesses during the period of gestation, including twelve (n=12; 7.41%) mothers reporting a viral infection during their first trimester. Four (n=4; 2.47%) had a preexisting history of diabetes mellitus and three (n=3; 1.85%) had hypertension diagnosed during the 1st trimester. Two (n=2; 1.23%) had epilepsy and one (n=1; 0.62%) mother had mumps during the first trimester of their pregnancy. Out of twenty two mothers with illnesses during their gestation, only fifteen mothers (n=15; 9.26%) had taken prescribed medicine for their illnesses in the first trimester. All four diabetic mothers had been treated with insulin therapy during the first trimester of their pregnancy. Sodium valproate and carbamazepine were the two drugs used by two epileptic mothers but the dose was not known. Eight (n=8; 4.94%) mothers had folic acid supplementation one month prior to the pregnancy and continued throughout the gestation.

Anthropometric measurements in less than five years old subjects

Anthropometric measurements of study subjects less than five years of age in the study sample were analysed. Weight, height, occipitofrontal circumference were recorded and following

parameters were calculated according to the age. There were eighty three (n=83; 51.23%) study subjects less than five years of age. Physical growth of these study subjects was calculated according to the guidelines issued by World Health Organization (WHO) to assess the physical growth and development of the children less than five years of age using WHO anthro (version 3.2.2.) software.

Weight for age measurements

The body weights of the study sample under five years of age ranged from 3kg to 19kg. According to the WHO guide lines (WHO anthro; version 3.2.2. software), there were sixteen (n=16; 19.28%) subjects with weight below the 3rd percentile for their age. Fifty three (n=53; 63.85%) subjects were between 3rd and 50th percentile and fourteen (n=14; 16.87%) subjects were above the 50th percentile for their weight for age (Table 7).

Height for age measurements

According to the WHO guide lines (WHO anthro; version 3.2.2. software), there were seventeen (n=17; 20.48%) subjects with height below the 3rd percentile for their age. Thirty eight (n=38; 45.78%) were between the 3rd and 50th percentile and twenty eight (n=28; 33.74%) subjects were above the 50th percentile for their height for age (Table 8).

Occipitofrontal circumference (OFC)

According to WHO guide lines (WHO anthro; version 3.2.2. software), eight (n=8; 9.64%) subjects had an OFC below the 3rd percentile for their age. Fifty (n=50; 60.24%) subjects were between the 3rd and 50th percentile and twenty five (n=25; 30.12%) were above the 50th percentile for their occipitofrontal circumference for their age (Table 9).

Body Mass Index (BMI)

The BMI of study subjects below five years of age ranged from 12 to 19. According to WHO guide lines, nineteen (n=19; 22.89%) were below the 3rd percentile, forty three (n=43; 51.81%) were between the 3rd and 50th percentile while twenty one (n=21; 25.30%) were above the 50th percentile (Table 10).

Table 7: Distribution of the weight for age among cleft palate subjects

Parameter	<50th percentile	%*	Expected %	>50th percentile	%*	Expected %	P value
Total	69	73.13	50%	14	16.8	50%	< 0.0001

(*Expressed as a percentage of the total number of subjects below five years old, n=83)

There was a statistically significant (one way chi-square test, P < 0.0001, chi-square 35.14) proportion of reduced weight for age among cleft palate subjects (Table 7).

Table 8: Distribution of height for age among cleft palate subjects

Parameter	<50th percentile	%*	Expected %	>50th percentile	%*	Expected %	P value
Total	55	66.26	50%	28	33.74	50%	0.0043

(*Expressed as a percentage of the total number of subjects below five years old, n=83)

There was a statistically significant (one way chi-square test, P < 0.01, chi-square 8.14) proportion reduced height for age among cleft palate subjects (Table 8).

Table 9: Distribution of OFC for age among cleft palate subjects

Parameter	<50th percentile	%*	Expected %	>50th percentile	%*	Expected %	P value
Total	58	69.88	50%	25	30.12	50%	0.0004

(*Expressed as a percentage of the total number of subjects below five years old, n=83)

There was a statistically significant (one way chi-square test, $P < 0.001$, chi-square 12.34) proportion of reduced OFC for age among cleft palate subjects (Table 9).

Table 10: Distribution of BMI for age among cleft palate subjects

Parameter	<50th percentile	%*	Expected %	>50th percentile	%*	Expected %	P value
Total	62	74.7	50%	21	25.3	50%	< 0.0001

(*Expressed as a percentage of the total number of subjects below five years old, n=83)

There was a statistically significant (One way chi-square test, $P < 0.0001$, Chi-square 19.28) proportion of reduced BMI for age among cleft palate subjects (Table 5.10).

Type of cleft palate

There were 125 (77.16%) subjects with cleft soft palate. Twenty-five (15.43%) had cleft palate involving hard palate. Bifid uvula was the next prevailing condition involving 7 (4.32%) subjects. Five (3.09%) subjects with submucous cleft palate were also found among these patients.

Associated clinical conditions

Prevalence of other clinical conditions of the study population was evaluated. Ninety-two (56.79%) subjects had associated other clinical abnormalities. Out of these, 58 (63.04%) were males and 34 (36.96%) were females.

Distribution of other congenital anomalies

Developmental delay and dysmorphic features were the commonest presentations occurring in 23 (14.2%) subjects each. The second most prevailing condition was cardiac malformation found in 15 (9.26%) subjects. Speech delay in 12 (7.07%), hearing and central nervous system abnormalities in 5 (3.09%) each, and epilepsy in 4 (2.47%) subjects were also noted. Genital, gastrointestinal, and renal anomalies were found in 2 (1.23%) subjects each. Visual abnormalities were seen in 1 (0.062%) subject (Figure 1).

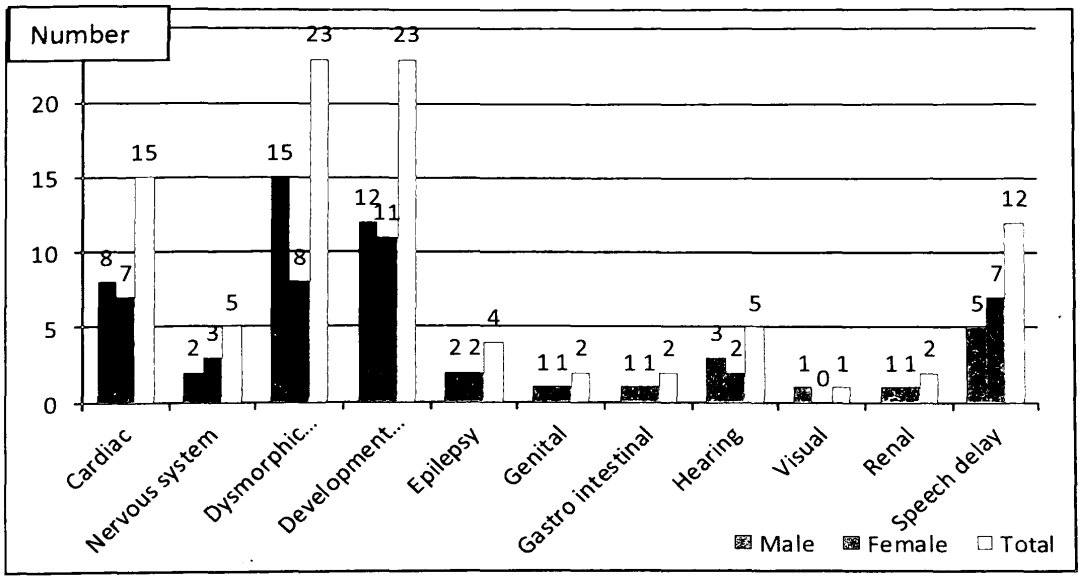


Figure 1: Types and distribution of other clinical conditions

Cardiac anomalies

Out of 15 subjects with congenital heart defects, 8 (53.33%) subjects with atrial septal defects (ASDs), 3 (20%) subjects with ventricular septal defects (VSDs), and 1 (6.67%) subject with Tetralogy of Fallot (TOF) were noted. Three subjects (20%) had either mitral valve prolapse (MVP), mitral stenosis (MS), or patent ductus arteriosus (PDA), or in combination.

Dysmorphic features

Dysmorphic features include abnormal facial features in 11 (47.83%) subjects, limb deformity in 6 (26.09%), and other minor abnormalities in another 6 (26.09%) subjects.

Developmental delay

Developmental delay including learning disability in 10 (43.48%), mild developmental delay in 9 (39.13%), and global developmental delay in 4 (17.39%) subjects was identified.

Psychological problem

Psychological problems were analysed separately and were found in 25 (15.43%) subjects with cleft palate. Of these, 17 (68%) were females and 8 (32%) were males. Fear to talk in the public was the commonest presentation and was seen in 17 (68%) subjects. Aggressive behavior in 4 (16%) and other minor psychological problems in 4 (16%) subjects were also identified.

Identification of 22q11.2 microdeletion in patients with cleft palate by PCR

A total of 162 patients with cleft palate were investigated by quantitative multiplex PCR for STS markers spanning the 22q11.2 region. All PCR products were analysed after agarose gel electrophoresis by using gel documentation system (Bio-Doc). There were no cases with 22q11.2 microdeletion identified (Figure 2).

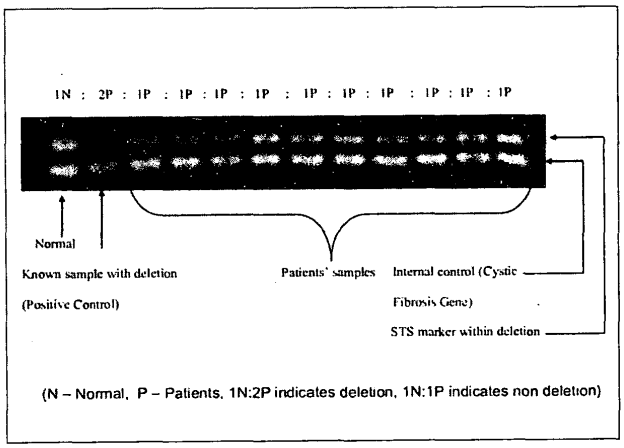


Figure 2: Dosage estimation of PCR products on 0.8% agarose gel (N = normal; P = patients; 1N:2P indicates deletion, 1N:1P indicates non-deletion)

Predictive value of the dysmorphic features

Out of one hundred sixty two (n=162) study subjects, only one hundred and sixty (n=160) subjects gave consent to allow photographs of their face, hands and feet to be taken. All the photographs of the study sample were reviewed by the researcher and a clinical geneticist who was blind to the results of semi-quantitative multiplex polymerase chain reaction investigation.

All the photographs were reviewed according to the guidelines formulated by Tobias *et al.*, (1999).

Subjects with characteristic dysmorphic features of the 22q11 deletion syndrome namely a broad bulbous nose with a square shaped tip of the nose, short philtrum, telecanthus, slanting eyes, hooded eye lids, low set ears, long slender fingers and hands were identified (Figure 3. A-D). There were seven (n=7; 4.37%) subjects with one or more characteristic dysmorphic features and identified as possible subjects with 22q11.2 deletion syndrome.

Figure 3: A-D Possible cases with facial features suggestive of a 22q11.2 deletion

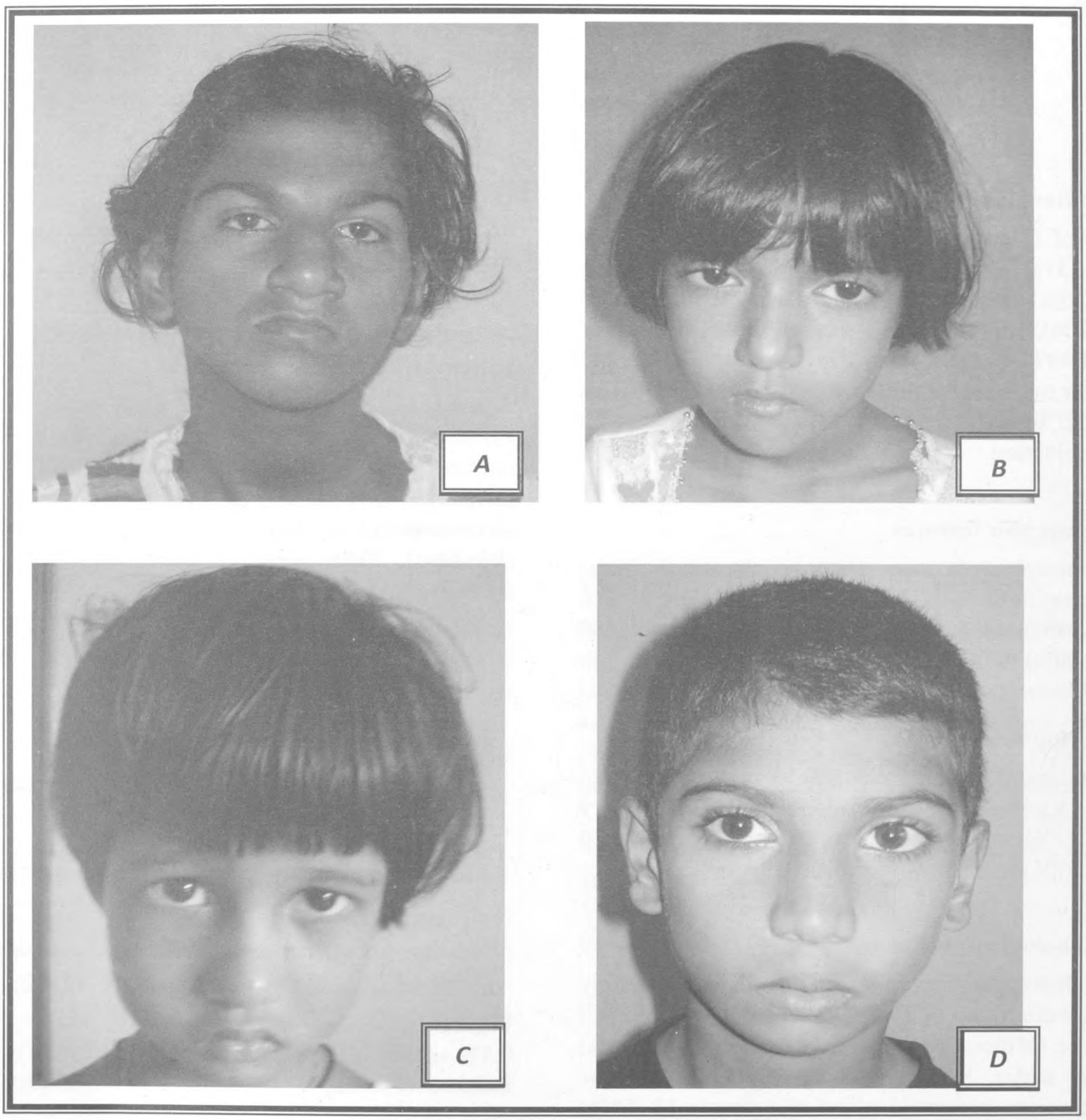


Figure 3A: Shows a thirteen year old female with a soft cleft palate and learning difficulties, bulbous nose tip, rectangular nose, hypertelorism, small mouth and short philtrum.

Figure 3B: Shows a seven year old female with a cleft soft palate and a family history of cleft palate. She also had a long and narrow face, small mouth, bulbous nose tip and rectangular nose

Figure 3C: Shows a two year old female with a cleft soft palate. She has a narrow face, bulbous nose tip, rectangular nose, hypertelorism, small mouth and hooded eye lids.

Figure 3 D: Shows a ten year old male with a cleft soft palate. This subject also demonstrated a narrow face, bulbous nose tip, rectangular nose and small mouth.

Syndromes associated with cleft palate

Some of the cases in this study had a clinically identifiable syndrome diagnosis and these included cases with a diagnosis of Goldenhar syndrome (Figure 4 A & B) in an eight year old male with microtia, pre auricular skin tags, epibulbar dermoid and submucus cleft palate. His mother and two brothers also had clinical features of Goldenhar syndrome. Apert syndrome was diagnosed in an eleven year old girl with craniosynostosis, hypertelorism, exophthalmia, non progressive hydrocephalus, cleft palate and syndactyly affecting her hands (Figure 5 A & B).

Suspected syndrome diagnoses included femoral hypoplasia and unusual face syndrome (Figure 6) in a four months old female with bilateral hypoplastic femurs causing severe leg shortening, short nose with a broad nasal tip, long philtrum, thin upper lip, micrognathia and cleft palate. Her mother did not have a history of diabetes mellitus or gestational diabetes mellitus. Fetal valproate syndrome (Figure 7) was a possible diagnosis in a five months old male with short and rounded palpebral fissures, bitemporal narrowing, thin upper lip, flat nasal bridge, prominent forehead, congenital heart defect, upward slanting eyes and cleft soft palate. His mother had taken sodium valproate before the conception and throughout the pregnancy as a treatment for epilepsy. A three months old male subject diagnosed with Pierre Robin sequence as he had glossoptosis and breathing difficulties (Figure 8 A & B).

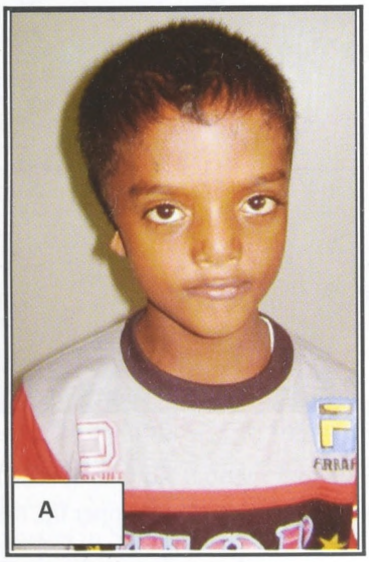


Figure: 4A Antero-posterior view

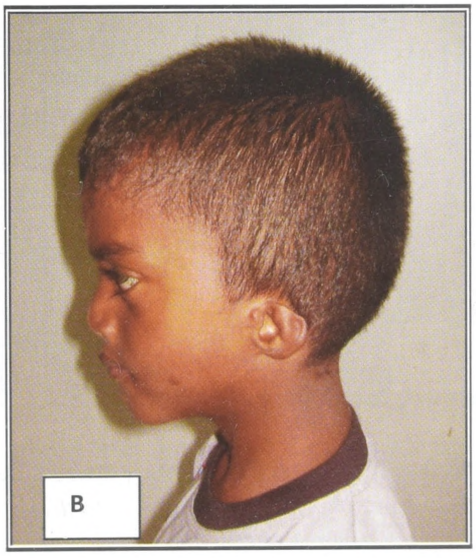


Figure: 4B Lateral view.

Goldenhar syndrome (Facio-auriculo-vertebral syndrome). An eight year old male microtia, preauricular skin tags, epibulbar dermoids and submucus cleft palate.

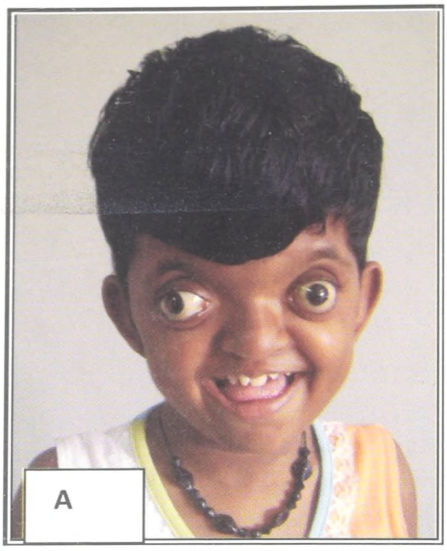


Figure 5A: Apert syndrome. Face of an eleven year old female with acrocephaly, hypertelorism, exophthalmia, progressive hydrocephalus and cleft palate.

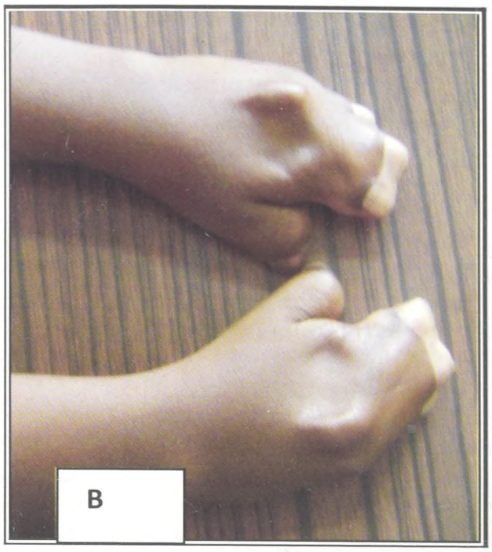
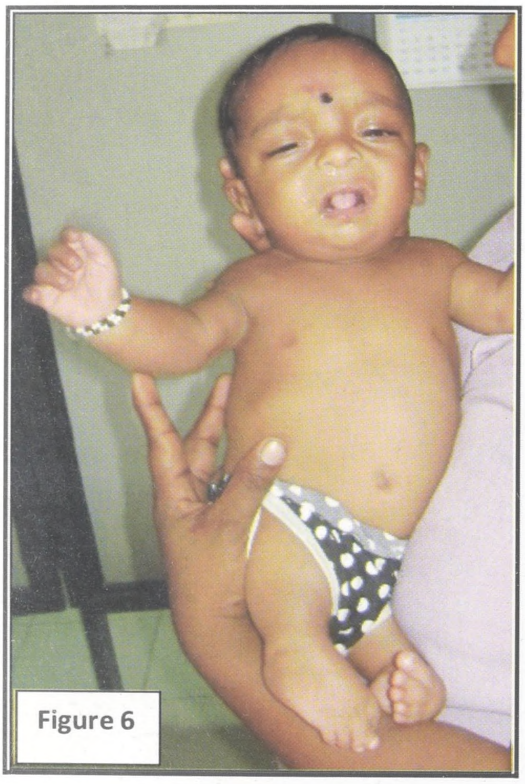


Figure 5B: Hands of an eleven year old subject with Apert syndrome showing syndactyly (fusion of fingers) and synonychia (fusion of nails)



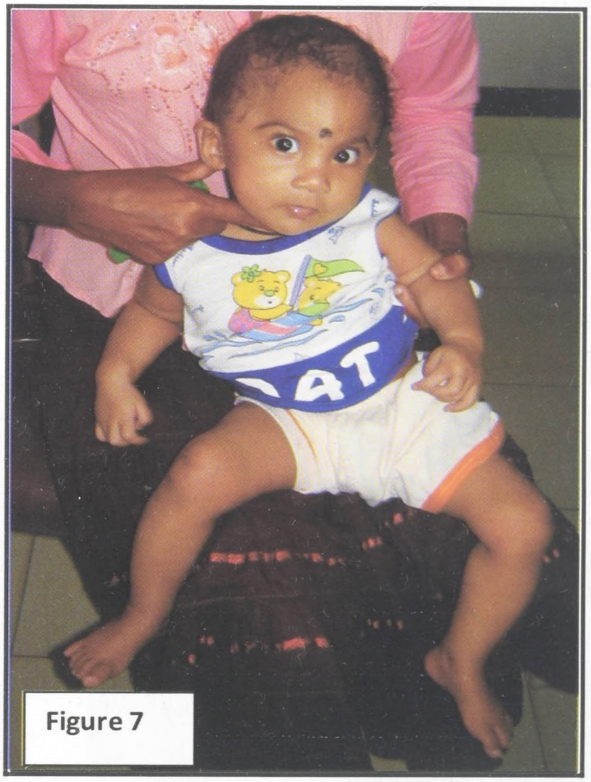
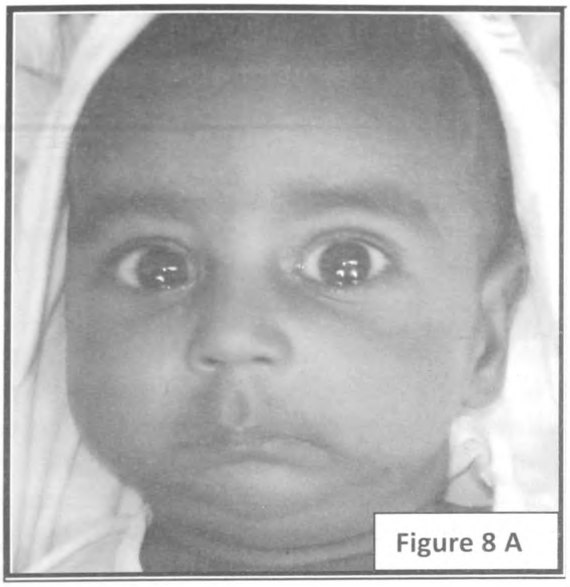


Figure 6: Possible case of femoral hypoplasia – unusual facial syndrome. A four months old female with severe femoral hypoplasia, bilateral club feet, short nose with a hooked nasal tip, long philtrum, thin upper lip, micrognathia and cleft palate.

Figure 7: Possible fetal valproate syndrome. This five months old boy had soft palate cleft, microtia, micrognathia, short nose with depressed bridge thin upper lip, bitemporal narrowing and metopic prominence, congenital heart defect and upward slanting eyes. His mother had epilepsy and was on sodium valproate before the conception and throughout the pregnancy.



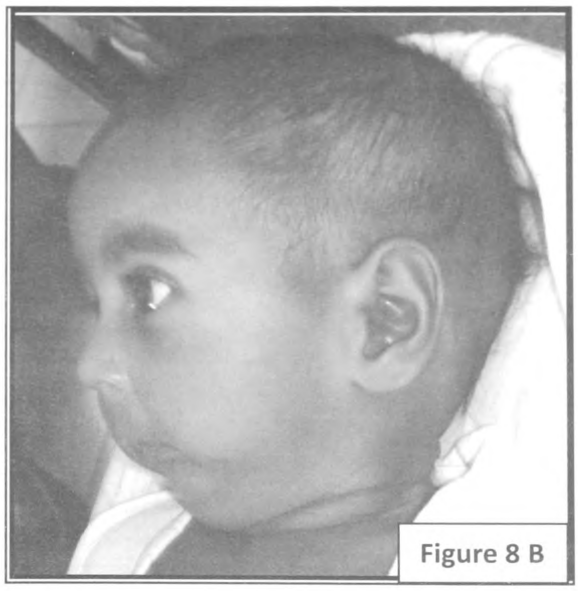


Figure 8A: AP view of the face

Figure 8B: Lateral view of the face of a three months old male diagnosed with Pierre Robin sequence. He had micrognathia, cleft in the soft palate, glossoptosis and mild breathing difficulties.

Discussion

Out of 323 subjects, 162 (50.15%) attended the clinic and participated in the study. Most of the patients had completed their surgical intervention, while few of them were waiting for their surgery.

Most of the patients (90.12%) were from the Southern Province of Sri Lanka. Majority (51.51%) were from Galle district while 24.78% were from Matara and 14.2% were from Hambantota districts. Patients from other districts including Kalutara (4.32%), Colombo (3.08%), and Ratnapura (2.48%) also participated in the study (Table 2).

In this group of 162 subjects with cleft palate, there were 95 (58.64%) females and 67 (41.36%) males. Male to female ratio (sex ratio) in the study sample and the original cleft palate population was 0.71 and 0.73 respectively. According to the department of census and statistics in Sri Lanka the sex ratio in the Southern province of Sri Lanka is 0.96 (Population and housing censuses in Sri Lanka, 2001). There was a significant different ($p = 0.0148$) in sex ratio of the study sample and the national figures (Southern province).

It shows that the cleft palate (without cleft lip) is more common in females than males in the Southern part of Sri Lanka (25) examined 593 patients with cleft lip and palate in Thailand and found female predominance in the cleft palate group. Cleft palate is more common in females in china (26) Australia (8) and Glasgow (27). In a study of 477 cleft palate patients in Jordan, Al-Omari and Al-Omari found that 74% of them were females, confirming the gender variation in cleft palate (28). In Estonia also, a study on epidemiologic factors causing cleft lip and palate shows that there is a female predominance in the occurrence of cleft palate (29). Results of all studies mentioned above are compatible with the results of the current study. Involvement of genetic factors such as X-linked recessive inheritance pattern has been explained by Rushton *et al, (*30). However, this is not in agreement with the results of above-mentioned studies as X-linked recessive inheritance is seen in male patients while females are only carriers (30).

In this current study sample, the majority of subjects were from Sinhalese families (93.2%) while 4.9% were from Moor families. This indicates a statistically significant higher incidence ($p = 0.007$)

of cleft palate among the Moor population of the Southern province of Sri Lanka. In a prospective and a retrospective evaluation of cleft palate patients in Kandy district, also concluded that there was a higher incidence of cleft palate among Moor subjects in Sri Lanka. Therefore, both studies suggest a higher prevalence of cleft palate among the Moor population in Sri Lanka although the reason for this is not known (9).

Birth positions in the family of all the one hundred and sixty two (n=162) subjects were analysed . Using data from the population and housing statistics, (2000), birth ranks of the Sri Lankan population were compared with the birth ranks of the current study. There was a statistically significant higher incidence ($p = 0.0001$) of cleft palate among first rank births in the Southern part of Sri Lanka.

In Sri Lanka, a study of etiological factors of five hundred and one (n=501) cleft lip and palate subjects showed, that there was no association between the birth rank and the incidence of cleft palate (45).

Martelli *et al.,* (2010) carried out a case control study (n=100 subjects each) to assess the environmental risk factors associated with cleft lip and palate, in a multi-professional reference service centre for craniofacial deformities in the state of Minas Gerais, Brazil, between 2006 to 2008. The frequencies of 1st, 2nd, 3rd, 4th and the 5th rank births in the case group were 50%, 24%, 12%, 9% and 5% respectively. Compared with controls there was no statistically significant association between the birth order and incidence of cleft lip and palate in the Brazilian population.[31]

In a case control study conducted by Ismail *et al.,* (2004), two hundred and one (n=201) non-syndromic cleft lip and palate subjects and two hundred and twelve (n=212) controls attending the outpatient clinic in the School of Dental Sciences, University of Sains Malaysia were studied. There was a significant rise in the incidence of cleft lip and cleft palate with the birth rank. Higher birth rank was shown to be associated with an increased risk of cleft lip and palate in this Malaysian population (46).

Studies in Malaysia (Boo and Arshad, 2002), Oxford, UK (Fraser and Calnan, 1960) and Cincinnati, USA (Bender, 2000), showed that higher birth rank was associated with an increased risk of cleft palate (39,47,48).

According to the human development unit of the South Asian region, the normal birth weight of a new born is defined as the birth weight between 2.5kg to 3.5kg. Below 2.5kg indicates the low birth weight (LBW) and birth weight below 1.5kg indicates the very low birth weight of the new born. A preterm birth is defined as a birth of a new born less than 36 weeks of gestational age (49).

Birth weight of a new born baby is an indicator of child's survival and its ability to bear the risks in early childhood. There are several common reasons for low birth weight in a newborn including maternal malnutrition, preterm delivery, maternal age at birth and multiple gestations. The Sri Lanka demographic and health survey (SLDHS), 2006/07 reported that 17.3% of live births had birth weight less than 2.5kg which falls in the category of low birth weight (Medical Statistics Unit, 2008). Incidences of low birth weight babies are higher in the estate sector than in rural and urban sectors in Sri Lanka (Medical Statistics Unit, 2008).

Nanayakkara *et al.,* (2011) conducted a prospective population based study involving four thousand one hundred and twenty (n=4120) births at the maternity unit, Teaching Hospital Kandy, in the years 2008 and 2009 to identify the birth weight of Sri Lankan newborns. The mean birth weight in the central province of Sri Lanka was identified as 2.854kg (50).

There was a higher proportion of LBW babies in the cleft palate population compared with the general population and the difference was which was statistically significant ($p < 0.0001$).

In a case control study of one hundred and forty two (n=142) cleft palate subjects (without cleft lip) in London, there was no statistically significant association ($p > 0.1$) between male subjects with cleft palate and birth weight. However, there was a significant association ($p < 0.02$) between low birth weight and being female cleft palate subjects (47). Wyszynski *et al.,* (2003) examined five hundred and eighty two (n=582) subjects with isolated cleft palate and healthy controls and reported that neonates with oral clefts are at higher risk of having low to very low birth weight. However, they found no association between the incidence of cleft palate and preterm births (51).

The peak incidence of cleft palate births was found in the month of March and the lowest incidence in the

month of October. This difference was statistically significant in both the highest (p=0.008) and the lowest (p=0.01) incidence months compared with the general population data (41).

Elliot *et al.,* (2008) evaluated all the cleft subjects that were operated during the period from 2000 to 2006 in nine provinces in Zambia. A total of four hundred and thirteen (n=413) cleft lip and palate subjects were evaluated and a statistically significant (p<0.01) increase in cleft births was observed in April and May. There was also a higher proportion of births between March to August (57.2%) compared to between September to February (42.8%) (6). Cervenka *et al.,* (1969) studied seven hundred and eighty one (n=781) cleft palate subjects for monthly incidence of cleft palate in Czech regions. A control group of normal subjects were also studied. There was a significantly higher birth incidence of cleft palate in February which was statistically significant when compared to the birth months of the general population (52).

Coupland and Coupland, (1988) studied three hundred and eighty one (n=381) patients with cleft palate in the Trent region, UK. Hospital activity analysis figures were used to obtain the birth dates of cleft palate subjects. Number of cleft palate births in the period from February to June was shown to fall two standard deviations or more below the annual mean births while in the period from July to October, the number of cleft palate births was more than two standard deviations above the annual mean. These results suggested that there was a seasonal variation in the incidence of cleft palate births in the Trent region (53).

Owens *et al.,* (1985) analysed the epidemiology of facial clefting in patients with cleft lip and palate notified to the Liverpool Congenital Malformations Registry during a period of twenty three years between 1960 and 1982. There were one hundred and fifty three (n=153) subjects with cleft palate among whom there was a significant trend towards an increase in the frequency of conception in the second half of the year (July to December) specially among female cleft palate subjects.

Qiao-Juan et al., (2006) investigated one thousand three hundred and thirty one (n=1331) patients who had a cleft palate repaired by the Smile Train program from 2000 to 2002. The variation of birth months demonstrated a significant seasonal trend with a peak incidence of cleft palate births in the summer (June to August) and lower incidence in winter (November to March) in China (54).

In a previous study of etiological factors for clefting among five hundred and one (n=501) cleft lip and palate subjects in Sri Lanka, a peak incidence of cleft palate was found in the month of April (9).

In a prospective and a retrospective evaluation of fifty one thousand five hundred and forty two (n=51542) live births and five thousand two hundred and sixty three (n=5263) stillbirths in the Kandy district of Sri Lanka by Amaratunga and Chandrasekera, (1989), there were five hundred and one (n=501) subjects with cleft lip with or without cleft palate and isolated cleft palate (55).

Physical growth of the cleft palate subjects below five years of age were assessed using a protocol stipulated by the World Health Organization (WHO anthro software version 3.2.2. January 2011). There were a total of eighty three study subjects below five years of age. Majority of them were below the 50th percentile for their weight for age, height for age, body mass index (BMI) for age and occipito-frontal circumference (OFC) for age and were statistically significant according to the WHO standards for the Asian population.

Ranalli and Mazaheri, (1975) in a case control study on two hundred and seventy nine (n=279) cleft palate subjects in Pennsylvania, USA and a control group of one hundred and seventy nine (n=179) matched subjects without cleft lip and palate evaluated the general physical growth of children with cleft lip and cleft palate using height and weight measurements. Their results showed that the weight and height for age of the cleft lip and palate children had no statistically significant difference from that of the normal control population. They had observed an early lag period of growth in the group of cleft lip and palate subjects. However, by three years of age these subjects were shown to reach their expected growth rates. These findings further confirmed the concept of catch up growth in small children (56).

Avedian, and Ruberg, (1980) reviewed and investigated weight records of thirty seven (n=37) infants with isolated cleft palate in Columbus, Ohio. Weights of these infants were plotted against a standard weight curve to determine the percentile

position of the weight of each study subject. The median birth weight of these subjects was at the 30th percentile. However, during the first five months of life, median weight was rapidly declined and persistently remained between the 20th and 25th percentile and started to catch up their growth at the age of six months (57).

Pandya *et al.,* (2001) studied one hundred and forty seven (n=147) infants with cleft lip and palate to identify the incidence of failure to thrive (FTT) during the period between 1993 and 1996 (120). Weights of each subject were analysed by using growth charts and standard deviation scores. There was a statistically significant higher incidence of failure to thrive in isolated cleft palate subjects (58).

Eighty three (n=83) children with cleft lip and cleft palate attending the outpatient cleft palate clinic at the Royal Victoria Infirmary, Newcastle were assessed by Lee *et al.,* (1997). The growth data were obtained and entered into the Castlemead growth program and transformed into standard deviation scores. The results were compared with the new United Kingdom national standard. All the cleft lip and palate subjects had normal birth weights. Lee *et al.,* (1997) concluded that there was a short term retardation of growth in children with clefts in the secondary palate. However, following cleft palate surgery, catch up growth was shown in these subjects without residual effect on either weight or attained height (59).

According to Hodgkinsons *et al.* (2005) in Northern Ireland, cleft in the secondary palate is commoner than the cleft in the primary palate (2) In Brazil, cleft soft palate is commoner (80%) than complete cleft palate.[31] According to the present study, the cleft soft palate is the commonest and there were only lesser number of patients with complete cleft involving the entire secondary palate and primary palate. These findings are found compatible with the results from Brazil and Northern Ireland (2,31).

Cleft palate is associated with lot of other major clinical anomalies. The published data vary significantly between studies. According to the available data, incidence of other associated anomalies varies from 2 to 55% worldwide (26,27,32). Survey of patients with cleft lip and palate in China shows lesser number of cleft palate patients (2.18%) associated with other clinical manifestations (26). An epidemiologic study of oral clefts in Iran showed 7.73% of cleft patients associated with other clinical manifestations, which is significantly higher when compared to the normal population (32). In Bulgaria, Vera Krumova (2008) found that there were 43.3% of cleft palate patients associated with other clinical malformations (29). According to the study of Boo *et al.* in 1990, 15.6% of cleft palate patients were associated with other clinical malformations, and in Estonia, 30.3% of patients with clefts had accompanying developmental anomalies (33,34).

Data from the Glasgow Register of Congenital Malformations were used to investigate the epidemiology of congenital facial clefts over the period 1974-1985 by Womersley and Stone in 1987. They found more than half of the infants (54%) with isolated cleft palate had other associated defects and noted that these anomalies were common in female cleft palate patients than males (61%) (27). In Scotland (35) identified that there was no significant association between gender and associated malformations in patients with cleft palate. This is not compatible with the results of this study where associated anomalies were common in males (54%) than females.

According to the Glasgow Register of Congenital Malformations, Pierre-Robin syndrome, musculo-skeletal anomalies, neural tube defects, chromo-somal abnormalities, and cardiovascular defects were the commonest defects associated with cleft palate (27). In Denmark, congenital heart defects, Pierre-Robin syndrome, Down syndrome, mandibulofacial dysostosis, anal atresia, Turner syndrome, Hirshsprung' disease, and chromosomal anomalies were the common clinical features associated with cleft palate (36).

Ruiter *et al.* in 2003 examined 99 patients with cleft palate and identified only one patient with 22q11 deletion among them and concluded that there is no justification for routine screening of 22q11 deletion in patients with cleft palate (37). According to Driscoll (38) the 22q11.2 deletion has not been found to be a cause of nonsyndromic cleft palate. Hence, prenatal testing is not recommended in the absence of other findings of 22q11 deletion syndrome. In this study, there were no patients found with 22q11 deletion among cleft palate subjects and it is compatible with the results of above-mentioned international studies.

Conclusion

Cleft soft palate is the commonest presentation of cleft palate and females are more prone to have cleft palate than males in Sri Lanka. Findings of this study further confirm the association of high incidence of congenital anomalies, developmental delays, dysmorphic features, and psychological problems in patients with cleft palate and reinforce the need of a high index of suspicion regarding the presence of such associated problems in cleft palate patients. Furthermore, it is advisable to search for syndromic diagnosis in patients with cleft palate. There is no justification for routine screening of patients with cleft palate for 22q11 deletion syndrome in Sri Lanka. It is advisable to formulate a guideline for screening of syndromic diagnosis and genetic investigation for cleft palate patients in Sri Lankan population.

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