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Original Article

Biochemistry Section

Reference Interval of Thyroxine and Thyroid Stimulating Hormone in Cord Blood in Tertiary Care Hospital, Kerala

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ABSTRACT

Introduction: Even though Congenital hypothyroidism (CH) is one of the most common causes of preventable mental retardation, diagnosis at birth is difficult due to absent or delayed clinical manifestations, owing to the transplacental transfer of maternal thyroid hormones. Delayed diagnosis and treatment can cause irreversible damage to the developing brain. The occurrence of cognitive dysfunction due to CH has reduced significantly in countries where Newborn Screening (NBS) is routinely practiced. Cord blood screening is considered to be an effective method to diagnose congenital hypothyroidism.

Aim: To establish reference interval for thyroxine (T3 and T4) and Thyroid Stimulating Hormone (TSH) in cord blood Medical College, Thrissur.

Materials and Methods: In the present cross-sectional observational hospital based study, the cord blood samples from 252 deliveries collected over a period of three months from May 2011 at in Government Medical College, Thrissur, Kerala, India. A 5 mL of cord blood samples were collected in a plain non vacuum tube with clot activator from umbilical cord incised 15-20 cm at the time of birth. T3, T4 and TSH was estimated by Enzyme Linked Immuno-sorbent Assay (ELISA) using kits of ELISA reader and Washer. Normality check done by visual inspection of histogram, Q-Q plot, box plot and Kolmogorov-Smirnov test.

Results: In total of 153 cord blood samples considered, and final analysis was done on 151 samples (69 males and 82 females) after excluding the samples of babies delivered by Lower Segment Cesarean Section (LSCS). The gestational age was mean 38.48 weeks and mean age of mothers was 24.22 years. More than 90% of the values for TSH is less than 12 mIU/mL and 90% of T4 values is less than 20 µg/dL. Reference interval determined by non parametric ascending rank order. Reference interval established is 0.63-17.033 mIU/L and 9.45-27.173 for TSH and T4 respectively, lower than those reported from studies in other parts of India. The mean value of TSH was 5.084 mIU/L and 87.5% of babies are having TSH values <10 mIU/L.

Conclusion: The study was conducted to establish reference intervals for cord blood TSH, T4 and T3 and confirms to the use of cut off values for the screening of CH for TSH as >20 mIU/L.

Keywords: Brain, Congenital hypothyroidism, Enzyme linked immunosorbent assay, New born screening

INTRODUCTION

Congenital Hypothyroidism (CH) is one of the most common causes of preventable mental retardation [1]. The role of thyroid hormone in the brain development is well established [2]. The clinical diagnosis of CH at birth is difficult since clinical manifestations are either obscure, absent or delayed owing to the transplacental transfer of maternal thyroid hormones [3]. Delayed diagnosis and treatment can cause irreversible damage to the developing brain. Neonatal Screening (NBS) for CH has thus proved to be beneficial clinically as well as in terms of cost effectiveness [4]. New born screening for CH was first started in Canada as early as 1972 and currently most of the developed countries had made it mandatory.

The occurrence of cognitive dysfunction due to CH has reduced significantly in countries where NBS is routinely practiced but 71% of the babies with CH are born in countries where there is no established NBS program [5]. The incidence of CH if found to be increasing worldwide after the initiation of NBS program and can be attributed mostly to early detection [6]. The incidence of CH in India varies from 1:2500 to 1:1000 according to studies conducted at various part of the country [7]. The incidence is found to be higher in southern states with a rate as high as 1:600-1:700 [8]. There is no nationwide screening program for CH in India, but few states like Goa and Kerala has implemented NBS program [9].

Even though, CH screening is widely accepted and practiced across the world, there is no universal agreement on the method of screening. Different strategies are followed with respect to the primary measurement, cut-off value, timing of blood collection and type of blood sample [5]. In most screening programs, blood samples collected on to the filter paper 24 hours after birth by heel prick or venous sample at three to six days is used for measuring thyroxine (T4) or thyroid stimulating hormone or both [10]. In developing countries like India, mothers and infants are often discharged early from the hospital and it is practically difficult to call back babies once they are discharged. In such settings cord blood screening is considered to be an effective alternative and is practiced in many Asian countries [11].

This study was done to establish reference interval for thyroxine and thyroid stimulating hormone in cord blood in Government Medical College, Thrissur. Since, hypothyroidism is one of the most common causes for preventable mental retardation, early diagnosis and treatment can prevent the progression of the clinical condition of such children and thereby reduce the burden of parents and the society.

MATERIALS AND METHODS

This study was a cross-sectional observational hospital based study conducted at Government Medical College, Thrissur, Kerala, India, after obtaining clearance from Institutional Ethic Committee. (Medical college Thrisur IRB communication document dated April 4, 2011). Cord blood samples from 153 consecutive deliveries were collected by convenient sampling over a period of three months from May 2011.

Sample size calculation: Determined according to the recommendations of the Clinical Labouratory Standards Institute

(CLSI) and International Federation of Clinical Chemistry (IFCC) which stipulates a minimum sample size of 120 to determine the 95th percentile reference limits (25 percentile and 97.5 percentile) by non-parametric method [12,13].

Inclusion criteria: All term babies born by normal vaginal delivery or caesarian section during the study time period were included in this study.

Exclusion criteria: Pre-term babies or those with less than 2.5 kgs of birth weight, or those with Apgar score <7 and babies born to mothers with antenatal history of thyroid diseases, pregnancy induced hypertenstion, gestational diabetes and any drug intake that can affect thyroid function were excluded from the study. Babies born to mothers with any co-morbidities and complications were excluded from the study affect thyroid function.

Study Procedure

Informed consent was taken from mothers. Details of mother was collected using a proforma which include age, parity, past history of illnesses, drug history, antenatal history etc. type of medication used during labour, anaesthetic agents used with dose and duration and type of antiseptic used on mother also were noted. After birth, baby's gender, birth weight and APGAR (Appearance (skin colour), Pulse (heart rate), Grimace (reflex irritability), Activity (muscle tone), and respiration) score at one minute was recorded.

A 5 mL of cord blood samples were collected in a plain non vaccum tube with clot activator from umbilical cord incised 15-20 cm at the time of birth. Samples are transported to the laboratory immediately for processing and stored at 20°C till analysis. T3, T4 and TSH was estimated by ELISA using kits from Diametra Italy in Biorad ELISA reader and Washer.

Principle of TSH Estimation

The TSH calibrators, patient specimens and/or controls containing the native antigen were added to streptavidin coated wells. Biotinylated monoclonal and enzyme labeled antibodies were added and the reactants mixed: these antibodies have high affinity and specificity and were detected against distinct and different epitopes of TSH. Reaction between the various TSH antibodies and native TSH occurs in the microwells without competition or steric hindrance forming a soluble sandwich complex. Simultaneously, the complex was deposited to the well though the high affinity reaction of streptavidin and biotinylated antibody. After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody bound fraction was directly proportional to the native antigen concentration. The activity of the enzyme present on the surface of the well was quantified by reaction with a suitable substrate to produce colour. By utilising several different calibrators of know antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

Principle of T4 Estimation

The T4 (antigen) in the sample competes with the antigenic T4 conjugated with Horseradish Peroxidase (HRP) for binding to the limited number of antibodies anti-T4 coated on the microplate (solid phase) (the enzyme conjugate should have no measurable binding to serum proteins especially Thyroxine Binding Globulin (TBG) and albumin). After incubation, the bound/free separation is performed by a simple solid-phase washing. Then, the enzyme HRP in the bound-fraction reacts with the substrate (H_2O_2) and the (3,3',5,5'-Tetramethylbenzidine) Substrate and develops a blue colour that changes into yellow when the Stop Solution (H_2SO_4) is added. The colour intensity is inversely proportional to the T4 concentration of in the sample. T4 concentration in the sample is calculated through a calibration curve.

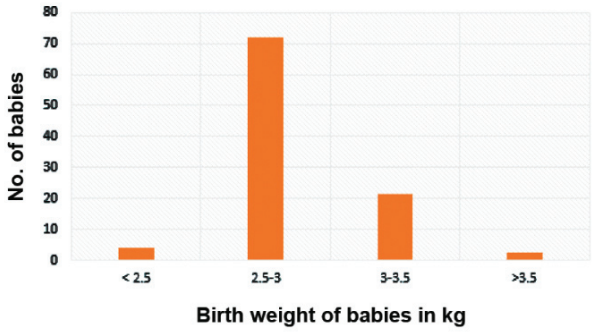
Quality control and quality assurance: Instrument calibration is done periodically according to the manufacture's instruction. Kit evaluation is done according to CLSI standards. Standard Operating procedure was prepared and followed for T4 and TSH estimation. Normal and pathological controls from Bio-Rad was used for every run.

STATISTICAL ANALYSIS

All data was entered into Microsoft excel and analysis was done using Statistical Package for Social Science (SPSS) trial version 20.0. Outliers were not removed for analysis. Normality check was done by visual inspection of histogram, Q-Q plot, box plot and Kolmogorov-Smirnov test.

RESULTS

One hundred and fifty one cord blood samples were analysed for T4 and TSH values after excluding the samples of babies born by LSCS. There were 69 males and 82 females. Birthweight ranged from 2.5 kg to 4.00 kg with a mean value of 2.8 kg and 72% of them weighed between 3.0 kg to 3.5 kg [Table/Fig-1,2]. APGAR score was noted at 1, 5, and 10 minutes. All Babies included in this study had APGAR score ≥7 at one minute. The distribution of birth weight is given in [Table/Fig-1]. Gestational age ranged between 37 to 42 weeks with a mean 38.48 weeks. The lowest maternal age was 18 years and the highest was 37 years with a mean age of 24.22 years. No correlation was obtained between cord blood T4 and TSH values and maternal age, type of delivery, gestational age, birth weight and sex of the baby.



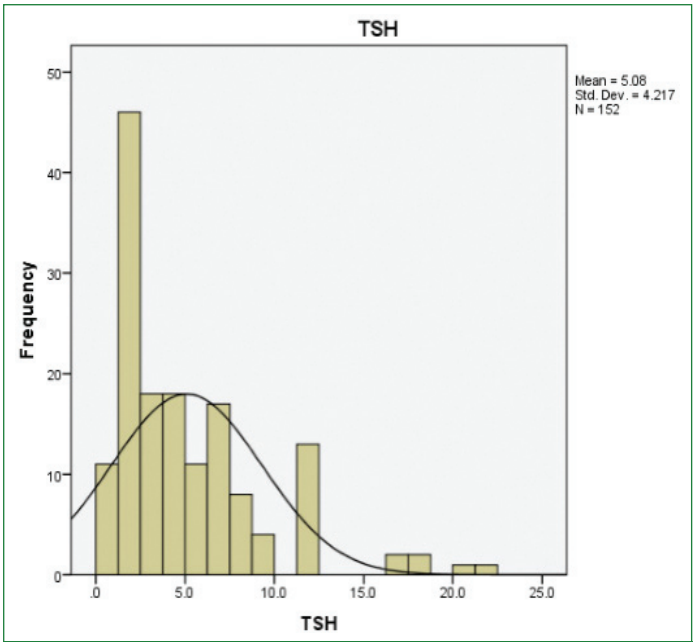
[Table/Fig-1]: The graph represents distribution of birth weight among the reference group.

Parameter	Minimum	Maximum	Mean	Median	Standard deviation
Maternal age (years)	18	37	24.22	23	4.272
Gestational age (weeks)	37	42	38.48	38.3	1.07
Birth weight (kg)	2.5	4	2.83	2.83	0.319
Cord blood TSH mIU/L	0.3	22.1	5.084	3.7	4.217
Cord blood T4 (µg/dL)	8.0	34	4.883	12.78	2.023

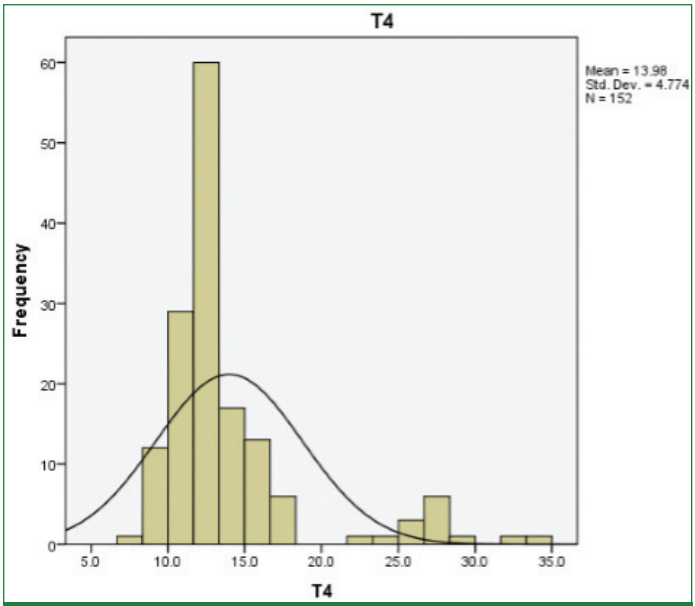
[Table/Fig-2]: Descriptive statistics.

Normality check for the data for T4 and TSH was done by histogram, Q-Q plot and box plot. Kolmogorov-Smirnov test was performed to further check the normality and the non gaussian distribution was found for both the parameters. The distribution of data for T4 and TSH is shown in [Table/Fig-3,4]. Both T4 and TSH data showed skewed distribution.

Reference interval [Table/Fig-5] was determined by non parametric ascending rank order statistics for determination of reference interval was followed as per recommendations of IFCC and CLSI guideline. A 95% confidence interval for both values was calculated by bootstrap method. More than 95% of the values for TSH is less than 12 mIU/L and ninety percentage of T4 values is less than 20 µg/dL [Table/Fig-6,7].



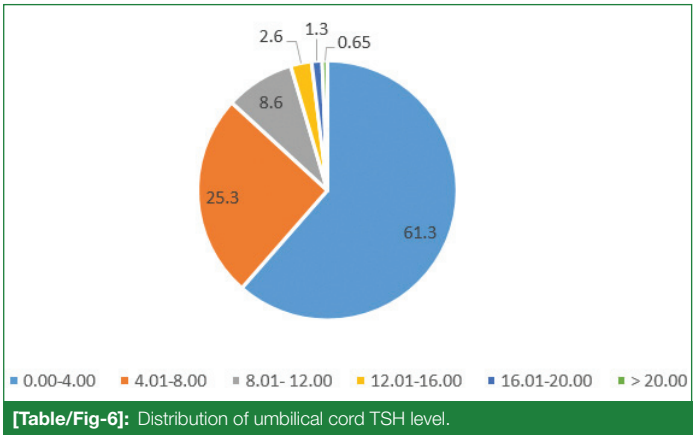
[Table/Fig-3]: Histogram showing skewed distribution of TSH levels in reference individuals.



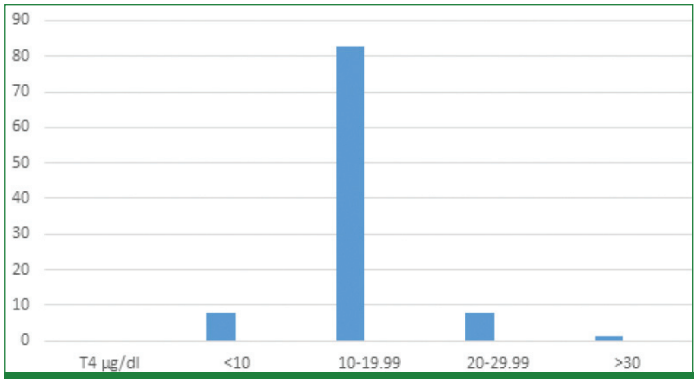
[Table/Fig-4]: Histogram showing skewed distribution of T4 levels in reference individuals.

Parameter	2.5th percentile	95% confidence interval	97.5th percentile	95% confidence interval
T4	9.45	8.413-9.735	27.173	27.070-34.564
TSH	0.63	0.300-1.171	17.033	12.300-20.863

[Table/Fig-5]: Reference interval for umbilical cord TSH and T4.
T4: Thyroxine; TSH: Thyroid stimulating hormone



[Table/Fig-6]: Distribution of umbilical cord TSH level.



[Table/Fig-7]: Distribution of umbilcal cord T4.

DISCUSSION

The authors decided the cut-off value of TSH as >20 mIU/L for recall, based on previous literature [14,15]. We had only two babies above the cut-off value. They were called back to test the venous sample for TSH and found to be within normal limit.

Successful implementation of NBS program has reduced the burden of CH in many developed and developing countries [2]. Unfortunately, a nationwide screening program in India is not introduced yet owing to many technical, financial and social factors [16]. Hence, the responsibility falls on the treating Paediatrician or Obstetrician to take initiative for the screening of all babies under their care [17]. But the screening guidelines and strategies vary widely across the countries [5]. Three screening methods used are primary T4, primary TSH screening or both. A primary T4 screening alone misses hypothyroid cases with normal T4. Primary TSH screening is widely accepted because primary hypothyroidism accounts for more than 90% of all cases. This strategy also has a disadvantage of missing the rare occurrence of hypothalamic pituitary hypothyroidism. The third strategy uses TSH and T4 as the primary test [18].

The cut-off value for TSH for screening also varies in different countries. Lowering the cut-off value has doubled the incidence of CH worldwide [19]. Lowering the cut-off also causes more false positives, increased recall rates, economic and labour burden. The cut-off values largely depend upon the time of sample collection. The time of sample collection for screening in various program differs. In many countries, NBS is performed at five to six days after birth to avoid the initial physiological surge at the time of delivery. A large number of healthy term babies are discharged early and recall of these babies for screening is practically difficult. Hence, many countries are opting for umbilical cord blood screening afterwards as an effective simple alternative [11,20,21]. Population specific reference intervals are an important pre requisite for better interpretation, diagnosis and treatment.

Reference interval established by this study is 0.63-17.033 mIU/L and 9.45 -27.173 µg/dL for TSH and T4 respectively which is comparable but the values are lower than those reported from studies in India by Soni LK, and Rani N and, and Jayachandran G et al., [22,23]. The mean value of TSH was 5.084 mIU/L which is also lower than that of other studies [24]. A 87.5% of babies are having TSH values <10mIU/L similar to other Indian studies [21,24].

The difference may be due to the difference in method of analysis or due to other epidemiological, geographical factors. Moreover, the present study has included only term babies with normal vaginal delivery with normal APGAR at one minute after birth. Babies born to mothers with any co-morbidities and complications are excluded from the study. Hence, the commonly observed factors those were likely to increase TSH levels, like preterm birth, other maternal and perinatal factors were omitted in this study.

Researchers all over the world have studied different TSH cut-off for CH screening with levels varying from 20-90, to minimise the false positivity the recall rate to make the screening more cost effective. In

Indian scenario TSH value of >20 mIU/mL is prove to be a safe cut-off which will not miss a true positive case [19,25]. In the present study, there were only two cases with TSH more than 20 mIU/dL which was later tested to be normal. Present study recall rate is 1.32% which is also lower at this cut-off point than the previous studies [21].

Those centers, employing primary T4 screening strategy is currently most of the screening program uses primary TSH screening due to high chance of false negative results especially in mild cases with normal T4 results. Conventionally, third percentile of total thyroxin was used as cut-off (<6 µg/dL). Few centers now use the 10th percentile (9 µg/dL) or 20th percentile. Higher the cut-off higher will be false positive rate and recall rate. In this study, we got the 3rd, 10th and 20th percentiles of T4 values as 9.450, 10.110 and 10.964 µg/dL, respectively which is much higher than the previous literature [26].

Limitation(s)

A ELISA is not a gold standard method for estimation of thyroid hormones, but in India, where most of the deliveries are occurring in primary healthcare system, by using a simple ELISA machine we can assess the thyroid hormone level of the new born babies at the primary healthcare system itself. We have included cord blood samples from babies delivered by full term normal vaginal delivery only in this study. So, effect of different maternal risk factors on thyroid functional status of the babies cannot be evaluated. Studies can be conducted to assess the effect of maternal risk factors on the thyroid function of babies can be conducted. Further, larger studies to assess the thyroid hormone level of babies of different gestational age group can also be done.

CONCLUSION(S)

The study was conducted to establish reference intervals for cord blood TSH, T4 and T3 in present hospital. The results obtained were 0.63-17.033 mIU/L and 9.45 -27.173 µg/dL for TSH and T4, respectively which is lower than those reported by other studies from India and other parts of the world for which geographic and ethnic factors may be attributed. The study confirms to the use of cut-off values for the screening of CH for TSH as >20 mIU/L. Raising the cut-off value may cause missing the diagnosis. However, setting up of cut-off value needs a larger study with more samples.

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