Introduction

Congenital Hypothyroidism (CH) is one of the common causes of mental retardation\(^1\) in children, which can be easily prevented if diagnosed within a few days after birth. Congenital hypothyroidism is a clinical condition when a baby is born with decreased thyroid hormone production at birth. Hypothyroidism in the newborn, may result from absence or abnormal development of the thyroid gland, destruction of thyroid gland in utero, failure of pituitary gland to stimulate thyroid gland, defective or abnormal formation of thyroid hormones. Of these, incomplete development of the thyroid gland is the most common defect. Most of the affected children would suffer from growth failure, irreversible mental retardation, and a variety of neuropsychological deficits\(^1,2,6\). Detection of CH, solely by clinical signs and symptoms alone is apparent at about three months, but by this time, irreversible damage to brain development would have already occurred. In North America and Europe, the prevalence of permanent CH is reported to be about 1:4000\(^3\). Girls are affected twice as often compared to boys. Realizing these facts, western countries have implemented National Level Screening Programs (NLSP) in the last two decades, to detect CH and institute early treatment. Similar efforts were instituted elsewhere in some countries in Eastern Europe, South America, Asia and Africa\(^2\). The World Health Organization (WHO) and the International Atomic Energy Agency (IAEA) have provided technical assistance\(^4\) and guidance to developing countries, to carryout pilot studies (see Fig. 1A-C). The efficacy of timely detection, treatment and its consequent benefits to the child is evident in Fig. 2 A-D. The Indian Pediatric Association (IPA)\(^5\) enumerates three main factors for the non-existence of NLSP in a developing country like India. They are: inconvenient methods, lack of reliable laboratories and cost.

A technically sound assay, suited for neonatal screening for CH that can be used by small and large laboratories throughout the country is an urgent requirement. Generally, TSH is assayed by ImmunoRadioMetric Assay (IRMA) or an equivalent non-isotopic assay in serum, for which about 1ml of blood is required. It is difficult to draw blood from 3-4 day old neonates and more so in pre-term babies and hence, serum-based TSH assays, as conducted on children and adults, would be difficult. Filter paper blood-spot assays are in use in many laboratories, world wide, but this technique has its own limitations\(^7,8\) i.e. collection and storage of samples on filter paper, effect of the type of filter paper used, uniformity in the spread of blood on the paper, variations in quantitatively eluting the TSH from the dried blood on the paper into the assay tubes, removing paper residue from the assay tubes during the washing step etc. All

\(^*\) Reasonably large screening studies from Hong Kong and Shandong province of China have shown an incidence of 1:2404 and 1:2831 respectively\(^9\).
Fig. 1A: IAEA and WHO disseminate technical know-how and conduct awareness programmes on Congenital Hypothyroidism.

Fig. 1B: Photographs from IAEA Documentation as seen in Figs. 1B&1C.

Fig. 1C: Photographs from IAEA Documentation as seen in Figs. 1B&1C.

Fig. 2A: Congenital hypothyroidism. An infant with cretinism. Note the hypotonic posture, coarse facial features and umbilical hernia.

Fig. 2B: Congenital hypothyroidism. Close-up of the face of the infant shown in Fig. 2A. Note the macroglossia.
these would contribute to making the analytical imprecision, quiet large. To reduce the imprecision we have used two approaches: 1) by extracting the blood from five blood-spot punches and using it for duplicate estimations and 2) carrying out the assay directly from a very small volume of whole blood i.e., 25 or 50μl. This volume of blood is much lesser than what is required for making blood spots on filter paper (Fig. 3A, 3B). With some practice, a semi-automatic pipette can be used to collect this small volume of blood directly from the heel-prick of an infant. We have compared the whole blood TSH assay with blood-spot TSH assays for assay performance and compared various assay parameters. Blood samples from adult thyroid patients, who visited our center for thyroid investigations, were used in the study and compared with serum TSH estimations carried out as per standard procedures.
Materials
Plain polystyrene test tubes (standard size 12 x 75 mm) were obtained from M/s. Tarsons, Kolkata. Tris buffer salt, EDTA, boric acid, sodium hydroxide were of Analytical Reagent grade and procured locally. Bovine serum albumin (BSA) was from Himedia, Mumbai. Whatman™ 3MM filter paper sheets (57 x 46 cm) procured locally were cut into strips of 7 x 23 cms. The strips were dried at 40°C for 2-3 h to remove adsorbed moisture if any, and wrapped in aluminum foil and were stored with desiccant sachets in self-sealing polyethylene pouches until required. A paper-punch to punch out 5mm spots from filter paper was procured locally. It is important that the punch is sharp and gives paper spots with clearly cut edges. hTSH IRMA kits used in the study were procured from BRIT, Vashi.

Methods

Preparation of Whole Blood Standards / QC for hTSH

Since the assay measures hTSH in whole blood, standards had to be prepared in whole blood. This is achieved practically by adding an equal volume of serum with known TSH-concentration to an equal volume of washed packed RBC, isolated from healthy donors so that 50% hematocrit is obtained as recommended by WHO. The procedure adopted is as follows: 7 ml blood from healthy volunteers without any thyroid dysfunction was drawn into EDTA Vacutainer™ tubes. RBC were separated by centrifuging at 3000 RPM for 10 min. and the plasma discarded. The RBC were washed 3 times by resuspending in 7 ml of sterile normal saline containing 0.2% sodium azide as preservative. Blood sample from different donors were treated separately to collect RBC. Packed RBCs from different volunteers were pooled and washed once with sterile normal saline. To 1ml of packed RBC placed in individual vials, 1ml each of hTSH serum standards 0, 5, 15, 50, 100 μIU/mL was added. Thorough mixing was ensured by swirling gently for several minutes. Similarly QC samples supplied with the kit were also adjusted to 50% hematocrit by adding RBCs. Whole blood standards were stored at 4°C and used within a month. Addition of RBCs to serum standards/QCs causes doubling of volume however, the TSH concentration remains the same as it is now expressed per ml of whole blood. Therefore, the results are directly reported in serum equivalent i.e. TSH μIU/mL serum.

Experiment-1: Blood Spot hTSH – IRMA

Preparation of blood spots for standards/QC/ samples on filter paper

Whole blood hTSH standard/QC (200 μL each) was drawn using semi-automatic pipette and spread on to Whatman filter paper strips as circles of about 2 cm diameter. Necessary precautions were taken to achieve uniformity in the spread of blood. A few such spots were made on each strip ensuring that there was sufficient space between spots to avoid overlap (Figs. 3A & B). Blood from adult patients was collected in plain glass tubes and 200 μL spotted immediately as described, before clotting took
The strips were made wide enough (7 cm) so that details of the patient, like Name, Case number etc. could be written on the strip itself. The strips were air-dried for an hour at RT then dried at 37°C for 2 h, wrapped in a clean aluminum foil and stored with a desiccant sachet in self-sealing polythene pouches at 4°C. Prior to setting up assays, the strips were brought to room temperature and 5 mm diameter spots were punched out from the blood spot choosing areas where spread of blood was uniform. These Filter Paper Blood Spots (FPBS) were used for assays as described below.

1) hTSH - IRMA using Two FPBS
In this experiment, two FPBS from each standard/QC/patient sample were placed in individual assay tubes appropriately labeled. 100μL of TSH tracer was added along with 100μL of Tris-BSA buffer (0.1 M with 0.02%BSA) to all the tubes. The tubes were incubated for 2 h in an orbital shaker at 150 RPM and then overnight (15 – 18 h) at RT without shaking. After incubation, contents of the tubes were aspirated, the filter paper spots removed carefully and the tubes washed twice with 2ml of wash buffer provided in the kit. The tubes were counted in a gamma counter for bound radioactivity. A calibration curve of TSH concentration vs bound counts was drawn. The sample values were read by interpolation from the calibration curve.

2) hTSH - IRMA using extract from Five FPBS
In this experiment, five FPBS from each standard/QC/patient sample were placed in individual plain polystyrene tubes labeled appropriately, 500μL of 0.05M phosphate buffered saline pH 7.4 (PBS) was added to each tube covered tightly and kept for elution overnight at RT on an orbital shaker (150 RPM). On the following day, the eluate was assayed for TSH as follows. 200μL of the eluate and 100μL of TSH tracer were added to the TSH assay tubes. The tubes were incubated for 2h at room temperature on an orbital shaker (150 RPM), after which, the tubes were aspirated, washed with wash buffer, counted for radioactivity and sample concentration obtained as stated earlier.

Experiment 2: Whole Blood hTSH – IRMA
Two sets of TSH antibody coated tubes were arranged and numbered from A – E along with tubes for samples and controls. 200μL of Tris-EDTA buffer with 0.02%BSA was added into all tubes. In one set, 25μL and in the other set, 50 μL of whole blood TSH standard/QC samples/patient samples were added. The tubes were incubated on an orbital shaker for 2h at RT. After incubation, the contents were aspirated out and tubes washed with 1ml of PBS. 100μL of TSH tracer and 200μL of PBS were then added to all tubes in both the sets. The tubes were further incubated for 1 h at RT on an orbital shaker (150 RPM). After incubation, the contents were aspirated and the tubes washed twice with wash buffer (0.1M borate buffer pH 8 containing Tween 20). The tubes were counted in a gamma counter for bound radioactivity and data processed as stated earlier.

Assay Validation
A) Recovery
Recovery samples were prepared by mixing 100μL of each whole blood standard A to E with 100μL aliquots of patient blood sample. These samples were assayed along with neat patients blood sample for calculating percentage recovery.

B) Correlation with serum TSH values
Patients samples (n = 69) were assayed for TSH by both, blood spot IRMA and whole blood IRMA and the values were compared with serum TSH. Statistical analysis performed and results tabulated.

Results
The results of all the experiments are summarized in Fig. 4 and Tables 1-3. Fig. 4 shows typical standard curves obtained with 2FPBS, 5FPBS, 25μL WB and 50μL WB. The assay with 50μL WB sample gives the standard curve with highest B_max and a better overall precision and this is expected as the sample volume is comparatively larger. A clear standard curve could be obtained with
the difference in bound counts between the various standard points in all the assay methods, though this is better with increasing sample volumes (Table 1). Among the assays compared, FPBS assays had higher imprecision compared to WB assays (Fig. 5). Recovery of TSH added to sample show

Table 1: Bound Counts (CPM) obtained in different hTSH assays

<table>
<thead>
<tr>
<th>Dilutions</th>
<th>Observed (TSH)</th>
<th>Corrected (D)</th>
<th>Expected (E)</th>
<th>Recovery %</th>
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<tr>
<td>Rabbits sample standardized</td>
<td>4.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sample + Standard (50mm) (10)</td>
<td>4.8</td>
<td>2.6</td>
<td>2.5</td>
<td>164%</td>
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<td>Sample + Standard (50mm) (10)</td>
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<td>7.5</td>
<td>7.5</td>
<td>100%</td>
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<tr>
<td>Sample + Standard (50mm) (10)</td>
<td>26.3</td>
<td>24.1</td>
<td>25</td>
<td>96.4%</td>
</tr>
<tr>
<td>Sample + Standard (100mm) (10)</td>
<td>53.3</td>
<td>51.1</td>
<td>50</td>
<td>162%</td>
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</tbody>
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Table 2: Recovery (50ml-Whole Blood hTSH IRMA)

Table 3: Correlation of Serum TSH with FPBS - TSH and WB - TSH

+ p< 0.01  * n = 69

Fig. 4: Comparison of standard curves from FBPS & WB TSH IRMA for neonatal screening

Fig. 5: Comparison of precision profiles from FBPS & WB TSH IRMA for neonatal screening
that it is within acceptable limits of ±15% over the entire range (Table 2). Table 3 gives the correlation coefficients observed between the TSH estimates (using serum samples in the standard lab procedure) and estimates using FPBS and WB samples.

**Discussion**

The assays were designed with standard points of 0, 5, 15, 50 and 100 μIU/ml since it is to be used for screening CH, where a moderate sensitivity is adequate, as the babies with TSH values >15 μIU/ml would be called back for follow up studies. The results show that it is possible to develop a TSH assay using either blood spots or small volumes of whole blood with existing TSH-IRMA kits*. Blood spot assays are reported to have a higher imprecision as compared to serum assays and were also observed in our study. The assay using 25μl and 50μl whole blood has better discrimination between various standards as compared to that of FPBS and also a lower %CV. The whole blood hTSH assay is simple, has minimum steps and short (3-4 h) assay time. All infants with TSH >40 μIU/mL are considered to have primary hypothyroidism until proved otherwise². Therefore, concentration region between 15 to 50 μIU/mL is of clinical importance and the count rate (CPM) obtained in this concentration region with WB format, is better as compared to that of FPBS. Automation of WB hTSH – IRMA would be easy whereas automating blood spot assay is difficult because the paper spots may block the aspiration probe of the machine.

A majority of European and Japanese programs favor screening by means of primary TSH measurements, supplemented by T4 determination on those infants with elevated TSH values, while CH screening programs practiced in North America use a two-tiered laboratory approach, in which, initial T4 measurement is followed by measurement of TSH in specimens with low T4 values. Diagnosis based on either one may miss out a variety of pathological situations². Screening for both T4 and TSH may not be feasible because of cost constraints. However, no treatment would be cheaper than prevention itself. The 5 FPBS assay protocol was considered seeing the replicate errors in the 2 FPBS assay. It requires an extra step of elution prior to assay but the reduction in imprecision and increase in confidence of the assay, makes up for the inconvenience. The concept of a WB assay was thought of, while spotting the blood from a heel prick onto a filter paper. If the heel is held up during the heel prick procedure, the blood wells up and forms a nice convex drop. It is quite easy to draw blood into a pipette directly from the heel prick into the assay tube. 25 μl or 50 μl samples for two or three replicates can be done quite quickly. Since the precision of the assay is quite satisfactory, repeat assays are seldom required. Nonetheless a third WB sample can be kept for repeat assay if required.

**Conclusion**

The major hurdle in screening for CH at national level in India appears to be the non-availability of a suitable TSH kit for neonatal screening. We have shown that it is

* For the purpose of standardization and validation, we have used blood spots and whole blood samples from adults, but since adult blood is not significantly different from neonatal blood vis-a-vis TSH measurements in the methods described would be suitable for neonatal blood also.
possible to use the existing hTSH-IRMA kit (supplied by BRIT) for neonatal screening for CH with minimum modifications. Depending on the convenience and availability of trained manpower, one can adapt either FPBS-TSH or WB-TSH from the heel prick of a three-day infant.

References