MATERIAL

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METHODS
MATERIAL AND METHODS

The study was conducted in the department of paediatrics with collaboration of department of microbiology M.L.B. Medical College, Jhansi.

SELECTION OF CASES: -

Study subjects comprised of children in age group of one month to 48 months suffering from severe systemic illness requiring intensive care.

Another group of patients consists of children weighting less than 50% of 50th percentile of Howard standard for their age. The cases of PEM were graded according to classification adopted by Indian academy of paediatrics.

The weight of patient was recorded nearest to 0.05 kg, by using infant weighting scale for weight of less than 10 kg, while adult type weighting machine was used in children weighting more than 10 kg, in the later weight was recorded nearest to 0.1kg.

SELECTION OF CONTROL:

Healthy children who matched for age and sex were taken from children of normal weight for age attending hospital for either checkup for minor ailments or brother and sister of patients admitted in the paediatric ward.

All children with maternal history of thyroid dysfunction, children with clinical evidence of endocrine abnormality, especially thyroid and infants with clinical goiter were excluded from the study.
All the cases were subjected to:

1. Detailed history regarding illness, relevant past history, family history, birth history and milestones.

2. Thorough clinical examination including anthropometric measurements.

3. Routine investigations and specific investigations as per requirement of illness.

4. Estimation of serum thyroid hormones.

**Specimen collection and preparation:**

For estimation of thyroid hormones, about 5 ml of blood was collected in plain vial without any additive through venipuncture after full aseptic precaution and blood was allowed to clot and serum was separated immediately by centrifugation.

Samples were stored for short time (up to 48hrs) at 2-8°C and for extended storage (up to 30 days) at – 20°C. Grossly hemolyzed, contaminated, lipemic samples and highly icteric samples were avoided. Repeated freezing and thawing were avoided. In case of frozen samples it was made sure to thaw samples completely before use.

**Estimation of serum thyroid hormones by ELISA method**

**Principle of estimation of triiodothyronine (T₃) and thyroxine (T₄)**

The ELISA test was performed as a solid phase competitive immunoassay. Microwells are coated with anti-T₃/T₄ antibody,
followed by blocking the unreacted sites to reduce non-specific bindings.

**Step 1** – T₃/T₄ antigen, present in samples and standards, and a fixed amount of T₃/T₄ conjugated with Horseradish Peroxidase compete for the binding sites of antiT₃/T₄ polyclonal antibodies coated onto the wells.

**Step 2** – The conjugated enzyme converts added substrate (TMB) to form a colored solution.

**Step 3** – The intensity of color change, which is inversely proportional to the concentration of T₃/T₄ in the samples are read by a microplate reader at 450 nm.

Results are expressed in ng/dl in case T₃ and µg/dl in case of T₄.

**Principle of estimation of serum thyroid stimulating hormone (TSH)**

The ELISA is performed as an indirect solid phase sandwich – type immunoassay, streptavidin – Boitin Method.

**Step 1** – Antigen present in controls, calibrators and patient sample binds to polyclonal anti TSH Enzyme labeled antibody and which vise versa binds irreversibly to the streptavidin–coated wells. The Bontinylated–Enzyme labeled antibodies exhibit different and distinct epitope recognition, reaction results between native antigen and the antibodies without competition or steric hindrance to form a soluble sandwich complex.

**Step 2** – The conjugated Enzyme converts added substrate (TMB) to from a colored solution.
Step 3- The intensity of color change, which is proportional to the concentration of TSH in the sample is read by a microplate reader at 450 nm. Results are expressed in μIU/ml.

REAGENT PREPARATION

1. Wash Buffer

   Wash buffer concentrate was diluted a final volume of 1000ml with distilled water in a suitable container prior to use.

2. Working reagent 1- T₃/T₄ Enzyme Conjugate solution

   Triiodothyronine–enzyme conjugate or thyroxine enzyme conjugate was diluted with triiodothyronine enzyme conjugate buffer or thyroxine enzyme conjugate buffer respectively in ratio of 1:11 in suitable container. For example - 160 μl conjugate diluted with 1.6 ml of buffer for 16 wells (a slight excess of solution was made). This reagent was used within 24 hours for maximum performance of the assay.

   General Formula:-

   Amount of buffer required = Number of well x 0.1
   Quantity of enzyme conjugate necessary = # of wells 0.01
   i.e. = 16X0.1=1.6 ml enzyme conjugate buffer.
   16X0.01=0.16 ml (160μl) for enzyme conjugate.

3. Working Substrate Solution

   Amount or reagent needed was determined and prepared by mixing equal portions of substrate A and substrate B in a suitable container. For example, 1ml of A and 1 ml of B were added per eight well strips (a slight excess of solution was made). This working
substrate solution prepared was used with in one hour for maximum performance of assay.

TEST PROCEDURE

Before proceeding with the assay, all the reagents and specimens brought to room temperature (20-27°C).

1. Wash buffer was prepared.
2. Working reagent 1-T3/T4 – enzyme conjugate solution was prepared.
3. Working substrate solution was prepared immediately before use.
4. Microplate wells for each serum reference, control and patient specimen were assayed in duplicate. Unused microwell strips were replaced back into the sealed pouch and stored at -20°C.
5. 50 µl for T3 and TSH and 25 µl for T4 of the appropriate serum reference, control or specimen was pipetted into the assigned well.
6. 100µl of conjugate was added to each well, bubble formation was avoided.
7. The plate was shaked thoroughly for 20-30 seconds.
8. Above microplate wells were incubated for 60 minutes for T3 and T4, and 120 minutes for TSH at room temperature.
9. Contents of the microplate wells discarded by decantation or aspiration. Then plates were dried by tapping and blotting with absorbent paper.
10. 300µl of wash buffer was added, decanted or aspirated. This procedure was repeated two additional times for a total of three (3) washes. A manual plate washer was used.
11. 100μl of working substrate solution was added to each well in the same order and timing of the conjugate.
12. Microplate wells were incubated at room temperature for fifteen (15) minutes.
13. 50μl stop solution was added to each well using the same order and timing as for the addition of the enzyme substrate.
14. Absorbance was read in each well at 450 nm in a microplate reader. The results were read within thirty (30) minutes of adding the stop solution.

Results

A dose response curve was used to ascertain the concentration of T₃/T₄ and TSH levels in unknown specimens.
1. Absorbance was recorded from the printout of the microplate reader.
2. Absorbance were plotted for each serum references, versus the corresponding T₃, T₄ and TSH concentrations in ng/dl for T₃, μg/dl for T₄ and μlui/ml for TSH on a linear graph paper.
3. Best-fit curve was drawn through the plotted points.
4. For determining the concentrations of T₃, T₄ & TSH for unknown, the average absorbance of the cases was located on the vertical axis of the graph and the intersecting point was obtained on the curve, and the concentration was read from the horizontal axis of the graph.

Limitations of the procedure

The assay should not be performed on grossly hemolyzed, microbially contaminated or lipemic samples.