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Review

The development and role of international biological reference materials in the diagnosis of anaemia

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ABSTRACT

Anaemia is a major global health problem. Although the main cause is iron deficiency, anaemia also results from other nutritional deficiencies (folate and vitamin B_{12}), haemolytic disorders including haemoglobinopathies, and bone marrow disorders. Accurate diagnosis of anaemia is dependent on reliable diagnostic tests and reference ranges, which in turn are dependent on effective standardisation. Standardisation is achieved through the availability of reference materials and reference measurement procedures. International biological reference materials have therefore been developed to standardise and control diagnostic tests for anaemia for a diverse range of analytes including total haemoglobin and haemoglobin types, ferritin, the serum transferrin receptor, serum vitamin B_{12} and folate, whole blood folate, and alloantibodies which mediate immune haemolytic anaemia.

1. Introduction

Anaemia, from the ancient Greek for 'without blood' is a condition defined today as a qualitative or quantitative reduction in the concentration of haemoglobin below a defined age- and sex-specific level (Table 1, modified from [1]). This results in a reduced oxygen-carrying capacity of the blood, leading to hypoxia in the organs. The decrease in haemoglobin concentration may be accompanied by a reduction in the red blood cell (RBC) count and the haematocrit. In its severe form, anaemia is associated with fatigue, weakness, dizziness and drowsiness, and may impact negatively on children's mental and physical development. Anaemias may be classified according to the cause: those caused by reduced production of haemoglobin and/or erythrocytes e.g. through dietary deficiencies or inherited haemoglobinopathies, and those caused by increased destruction or loss of red cells (Table 2).

The World Health Organization (WHO) has estimated that around two billion people worldwide are anaemic. In view of the scale of this global health problem, efforts have been made to improve the accuracy of diagnosis of anaemia and the assessment of the iron status of populations. This review will focus on the development of biological reference materials in achieving these aims (Table 2).

2. Standardisation of haemoglobin measurement

Fundamental to the diagnosis of anaemia is accurate measurement of the haemoglobin (Hb) concentration. The availability of reference materials and methods are therefore critical for the standardisation and validation of Hb measurement.

Haemiglobincyanide (HiCN; cyanmethaemoglobin) is a stable haemoglobin derivative into which virtually all haemoglobin types can be easily converted using appropriate cyanide–ferricyanide reagents [2,3]. In 1966, the spectrophotometric determination of HiCN was accepted as the international method of choice for the determination of Hb concentration in human blood [4], as several different methods were in use which gave widely discrepant results. Recommendations for the preparation and specifications of HiCN reference solutions were developed [5] which have since been subjected to regular revisions [6–8]. The most recent recommendations state that the Hb concentration should be calculated from the spectrophotometric measurement of HiCN absorbance at 540 nm using a millimolar absorptivity of 11.0, the purity should be checked by the ratio of absorbance at 540 nm and 504 nm (which should lie between 1.59 and 1.63), and the turbidity should be checked by measurement of absorbance at 750 nm (which should be $\leq$0.003 per cm lightpath length).

Since the first reference standard for HiCN was established in 1966 by the International Committee for Standardization in Haematology (ICSH), and given 1st International Standard (IS) status in 1968 by the WHO, five successive lots of HiCN have been prepared and issued [reviewed in 9]. The current WHO reference

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Table 1
The World Health Organization's haemoglobin thresholds used to define anaemia.[a]

Age or gender group	Hb threshold (g/dL)
Children (0.5–5.0 yrs)	11.0
Children (5–12 yrs)	11.5
Children (12–15 yrs)	12.0
Women, non-pregnant (>15yrs)	12.0
Women, pregnant	11.0
Men (>15yrs)	13.0

[a] Modified from [1].

material, designated the 6th IS for HiCN and established in 1998, is a portion of a batch produced for the European Commission Bureau of Reference (BCR) and held at the Institute for Reference Materials and Measurements as CRM522. The preparation was derived from lysed, purified bovine haemoglobin converted to HiCN by addition of a cyanide–ferricyanide reagent, and sealed in brown neutral borosilicate glass ampoules. Although the earlier reference solutions consisted of human HiCN, which appears generally stable for about 15 years [10], bovine HiCN was chosen for the 6th IS as it appeared to be even more stable than human HiCN, and, at the time, it was considered safer to use bovine rather than human material. The mean absorbance at 540 nm of the HiCN solution from 156 individual measurements from 13 laboratories in Europe and the USA was 0.5457. However, while BCR based their mass concentration assignment of 800.3 mg/L on an extinction coefficient at this wavelength of 11.0 L $mmol^{-1}$ cm^{-1} [7], WHO used an extinction coefficient of 10.96 L$mmol^{-1}$ cm^{-1}, determined by iron analysis, to assign a value of 803.3 mg/L [11]. Both BCR and WHO used a relative molecular mass of the bovine Hb monomer of 16,133. Nevertheless, the discrepancy in value assignments may be considered insignificant. The standard is intended to be used to calibrate the function of spectrophotometers at 540 nm, and to check the validity of manufactured preparations of HiCN that are used as secondary standards for internal quality control of spectrophotometers used in routine haemoglobinometry. The standard may also be used to prepare calibration graphs in order to convert absorbance measurement into haemoglobin concentration.

3. Anaemia due to iron deficiency

Most of the normal body iron content (3–5 g) is haemoglobin iron, and iron deficiency anaemia (IDA) is the most common type of anaemia, occurring widely in both developed and developing countries. Advanced iron deficiency and the subsequent reduction in haemoglobin production is characterised by the presence of hypochromic and microcytic red cells. Pregnant women and young children are the most susceptible to iron deficiency anaemia; there are almost no countries where anaemia is not at least a mild public health problem in these population groups [1]. Iron deficiency usually results from inadequate dietary intake, and it often co-exists with other conditions associated with anaemia such as gastrointestinal parasitic infections and haemoglobinopathies. In adult males, iron deficiency is often due to bleeding from the gut due to bowel cancer and should always be thoroughly investigated. Although serum iron, total iron binding capacity (TIBC), and calculation of the percent transferrin saturation are all used as diagnostic parameters in the evaluation of iron deficiency (as well as iron overload), satisfactory reference materials and methods are not available for these measurement procedures. Other key indicators of iron status for which reference materials have been produced include measurement of the concentrations of serum ferritin and the serum transferrin receptor.

3.1. Ferritin

Ferritin is the major iron storage protein occurring in all body cells and in body fluids, keeping the iron in a soluble and non-toxic form, and releasing it in a controlled way. The concentration of serum ferritin correlates well with the total iron stores if there is no concurrent infection, and, following the description of an immunoradiometric assay for serum ferritin in 1972 [12], the immunoassay of serum ferritin has become widely used in diagnosing iron-related disorders such as anaemia, and in population surveys of iron status. Such assays are 'sandwich' immunoassays in which one antibody 'captures' the target analyte in the sample, and another antibody, conjugated to an enzyme now rather than a radioisotope, is the 'detecting' antibody. The enzyme label generates a colour or light signal that is proportional to the amount of analyte present in the original sample. The antibodies can be polyclonal and/or monoclonal and they determine the epitope specificity of the assay.

Ferritin is an almost spherical water-soluble complex composed of 24 subunits, which exists in several distinct forms or isoferritins. The heterogeneity arises from the presence of varying proportions of two subunit types, H and L chains, which are genetically distinct and share about 50% sequence homology [reviewed in 13]. For example, an L-rich form predominates in serum, liver and spleen whereas heart tissue contains an H-rich form. This heterogeneity results in immunological differences [14]. Within the protein (apoferritin) shell, iron is stored in the Fe(III) oxidation state incorporated in the mineral ferrihydrite, $[FeO(OH)]_8[FeO(H_2PO_4)]$, which is attached to the inner wall of the shell. Apoferritin can store ~ 4500 iron-containing molecules in this way.

3.1.1. Standardisation of immunoassays for serum ferritin

During the 1980's, more laboratories were carrying out ferritin immunoassays using a range of techniques and in-house standards prepared from liver or spleen, which were the only practicable sources of ferritin at that time. The differences in ferritin preparation and possible selection of different ferritin isoforms combined with the inter-laboratory variability in serum ferritin estimates prompted the ICSH to undertake the preparation and characterisation of human ferritin for use as a common standard [15]. Ferritin was prepared from normal human liver obtained post-mortem, or from spleen from splenectomised patients with transfusional iron-overload using several common steps and either ultracentrifugation to isolate iron-rich ferritin, or repeated crystallisation from 5% cadmium sulphate. A number of preparations were characterised, and two were lyophilised using human serum albumin as excipient. One lyophilisate, coded 80/602 at the National Institute for Biological Standards and Control (NIBSC), contained normal liver ferritin, the other lyophilisate, coded 80/578, contained iron-rich spleen ferritin. However, the two preparations were not immunologically distinguishable in the majority of immunoassays for serum ferritin, both demonstrated satisfactory stability, and both were deemed suitable for use as reference materials for the assay of serum ferritin. Preparation 80/602 was subsequently established as the 1st IS for ferritin by the WHO in 1985 [16]; upon exhaustion of the stocks, it was replaced by the spleen ferritin lyophilisate 80/578 as the 2nd IS in 1992 [17]. Both preparations were assigned values of 9.3 μg/ampoule, based on the ferritin protein content determined using the method of Lowry [15]. Both preparations were widely used to calibrate working standards and immunoassays, including commercial kits, thereby improving the comparability of results from different methods such that serum ferritin levels below 12–15 μg/L are indicative of depleted iron stores [18].

In the mid-1990's, when stocks of the 2nd IS were dwindling, it was decided to evaluate a recombinant ferritin preparation of L subunits as a potential replacement [19–22]. This was due to