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# Comparison of haemoglobin H inclusion bodies with embryonic ζ globin in screening for α thalassaemia

L C Chan, J C C So, D H K Chui

**Abstract**

***Aims*—To compare the haemoglobin (Hb) H inclusion test with immunocytochemical detection of embryonic ζ chains in screening for α thalassaemia.**

***Methods*—Blood samples from 115 patients with relevant clinical history and hypochromic microcytic indexes were screened using the HbH inclusion test and the Variant Hemoglobin Testing System (BioRad, Hercules, CA, USA).**

***Results*—The HbH inclusion test was positive in 61 of 115 cases, three of whom had HbH disease confirmed by electrophoresis. The remaining 58 had α thalassaemia 1. All three HbH cases and 56 of 58 cases of α thalassaemia 1 expressed embryonic ζ chains, giving a specificity of 96·7%. Fifty four of 115 cases had a negative HbH inclusion test, of whom 50 had β thalassaemia trait and three had iron deficiency. No diagnosis was reached for the remaining patient.**

***Conclusion*—The immunocytochemical test is as sensitive as the HbH inclusion test in screening for α thalassaemia. The presence of ζ chains is highly specific for α thalassaemia 1 incorporating the (--/SEA) deletion. The specificity and simplicity of the immunocytochemical test make it the test of choice in screening for α thalassaemia.**

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Keywords: HbH inclusion test, ζ chains, α thalassaemia.

In South East Asia, where α and β thalassaemia are prevalent, the haemoglobin (Hb) H inclusion test, based on incubation of erythrocytes in brilliant cresyl blue, is used extensively in many laboratories as a screening test for subjects with α thalassaemia. The presence of HbH inclusions is correlated with the αα/-- genotype (including those with deletions involving the complete ζ-α gene cluster) as present in α thalassaemia 1 carriers and the α-/-- genotype associated with HbH disease.[1] Based on positive findings, couples at risk of producing fetuses with hydrops fetalis due to Hb Barts are identified for genetic counselling and prenatal diagnosis. Recently, an immunocytochemical test which detects embryonic ζ chain in erythrocytes was shown to be highly specific and sensitive for α thalassaemia 1 carriers of the (--SEA/) deletion[2]—that is, the αα/-- genotype in which the ζ globin gene cluster is intact. We have compared the HbH inclusion test with immunocytochemical detection of ζ globin chains in screening for α thalassaemia.

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## Methods

Blood samples from 115 patients with relevant clinical history and microcytic (mean corpuscular volume (MCV) < 80 fl) and hypochromic indexes were subjected to the following studies: (1) HbH inclusion test; (2) estimation of HbA2 and HbF by the Variant Hemoglobin Testing System[3] (BioRad, Hercules, California, USA); and (3) electrophoresis of the haemolysate in cellulose acetate at pH 8·55 using the Super Z kit Hb system as appropriate. Using the Variant Hemoglobin Testing System, the normal adult reference ranges for HbA2 and HbF in our laboratory are, respectively, 2·3–3·0% and 0–0·9% (unpublished data). The normal adult reference range for MCV in our local population, based on determination of erythrocyte indexes using the Technicon H*1 blood analyzer, is 83–96 fl.[4] Serum iron and serum ferritin concentrations were determined in 40 cases.

### HbH inclusion test

Air dried peripheral blood smears were made from a mixture of two parts 1% brilliant cresyl blue to one part blood following incubation at 37°C for exactly 30 minutes. For detection of inclusions, 1000 to 5000 red blood cells were examined under an oil immersion lens which requires up to 15 minutes per case.

### Detection of embryonic ζ chains

Air dried peripheral blood smears were fixed in a solution containing 90% absolute acetone and 10% absolute methanol at room temperature for five to 10 minutes. They were then washed in phosphate buffered saline (PBS) containing 3% bovine serum albumin (BSA); 10 ml of a 1 in 20 dilution of murine monoclonal antihuman ζ globin chain conjugated with fluorescein isothiocyanate was then added. The slides were incubated in a humidified chamber at room temperature for 30 minutes and were then washed in BSA free PBS, covered with glycerol and examined under a fluorescence microscope (Zeiss). Cord blood and blood from an individual with a normal full blood count were used as positive and negative controls, respectively, for ζ globin expression.

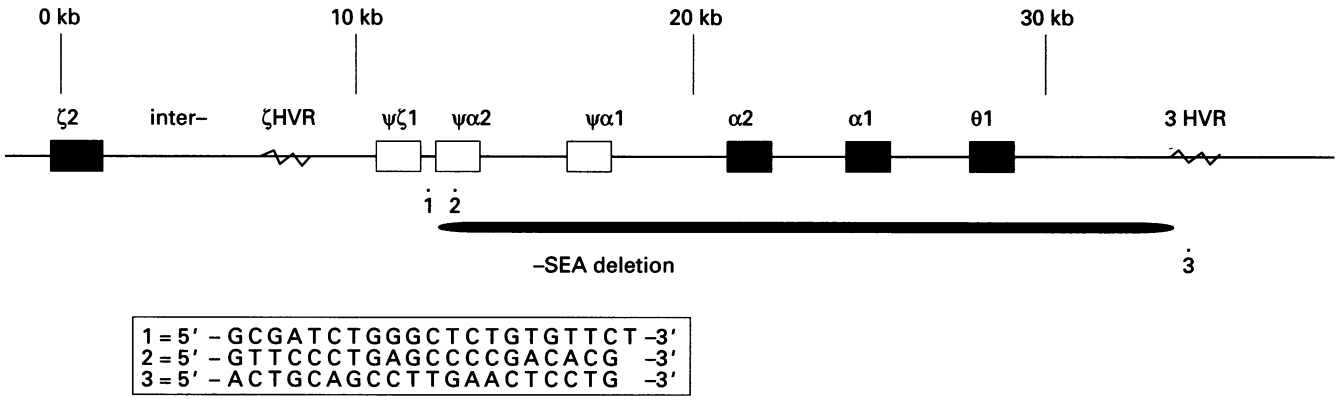


*Figure 1 Alpha globin gene loci and primers for PCR analysis.*

DNA EXTRACTION AND POLYMERASE CHAIN REACTION

DNA was extracted from peripheral blood using a commercial kit (Intragene Purification Matrix; BioRad). To detect the (--/SEA) deletion, three primers were synthesised using details of the sequences provided by Chang *et al*[5] (fig 1). Primers 1 and 2 amplify a 314 nucleotide band if normal or if α thalassaemia 2 is present, whereas α thalassaemia 1 with the (--/SEA) genotype will yield a 195 nucleotide band on amplification with primers 1 and 3. DNA (250 ng) was amplified using the polymerase chain reaction (PCR) with primers at a final concentration of 2 μM for 35 cycles on an automated themocycler under the following conditions: denaturation at 94°C for 30 seconds, annealing at 58°C for 45 seconds and extension at 72°C for one minute, except for the first cycle when denaturation took place at 95°C for 90 seconds and at 94°C for 30 seconds. After amplification, DNA was electrophoresed on a 2% agarose gel at 100 V for one hour and visualised using ethidium bromide.

STATISTICAL ANALYSIS

The sensitivity and specificity of the immunocytochemical test for the diagnosis of α thalassaemia were calculated as described by Simel.[6]

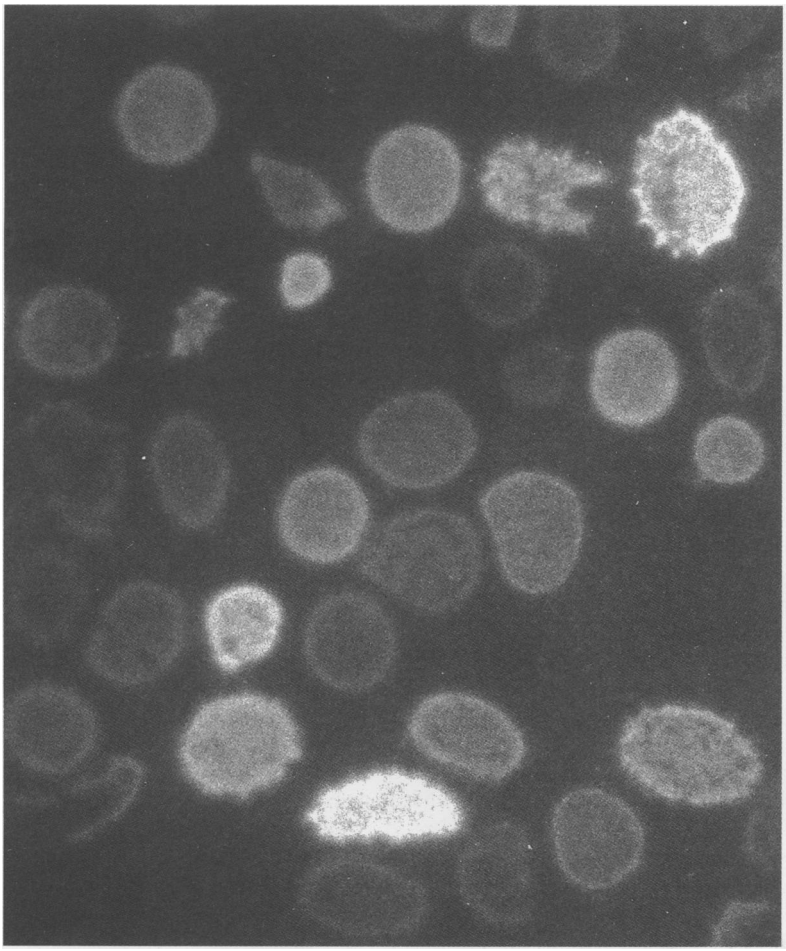

*Figure 2 Immunofluorescent erythrocytes stained with anti-ζ chain antibody in a case of α thalassaemia 1. (Magnification × 2000.)*

## Results

The HbH inclusion test was positive in 61 of the 115 cases studied. Three of these 61 patients had HbH disease based on the presence of multiple inclusions and a HbH band on electrophoresis. In the remaining 58 the pattern of HbH inclusions was compatible with a diagnosis of α thalassaemia 1. All three cases of HbH disease expressed embryonic ζ chains as did 56 of the 58 cases of α thalassaemia 1. Therefore, the sensitivity of the immunocytochemical test compared with conventional methods for the diagnosis of α thalassaemia was 96·7% (59/59+2). Figure 2 shows the fluorescence pattern of erythrocytes, revealing varying staining intensities in a patient with α thalassaemia 1.

Of the two patients (cases 47 and 71) with HbH inclusions, but without embryonic ζ chains, one had iron deficiency anaemia whilst the other had an unexplained hypochromic microcytic anaemia with no family history of thalassaemia. As expected, the (--/SEA) deletion was not detected on DNA analysis of the α globin locus by PCR in both of these cases. By contrast, an amplified band of 195 base pairs, confirming the presence of the (--/SEA) deletion, was present in all seven cases of α thalassaemia 1—that is, both HbH and ζ chain positive. The results of the DNA analysis are presented in fig 3.

Fifty four cases had a negative HbH inclusion test. Fifty had β thalassaemia trait based on raised A2 and F concentrations and three were

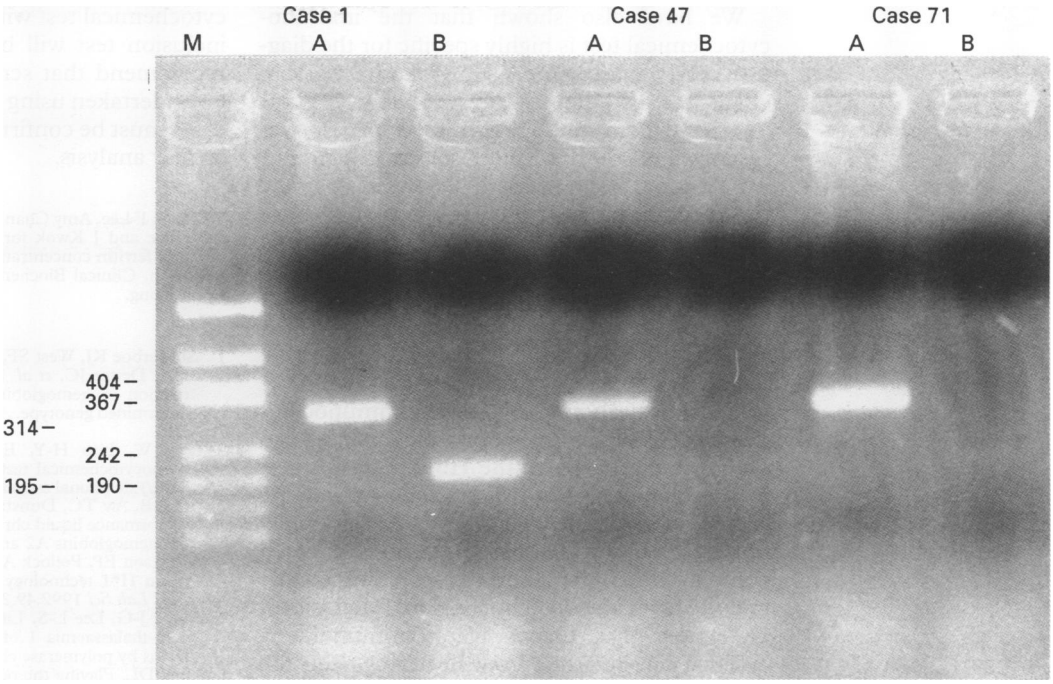


*Figure 3 An ethidium bromide stained agarose gel of DNA amplified from cases 1 (HbH positive, ζ chain positive), 47 and 71. Lane A, primers 1 and 2; lane B, primers 1 and 3; M, DNA markers.*

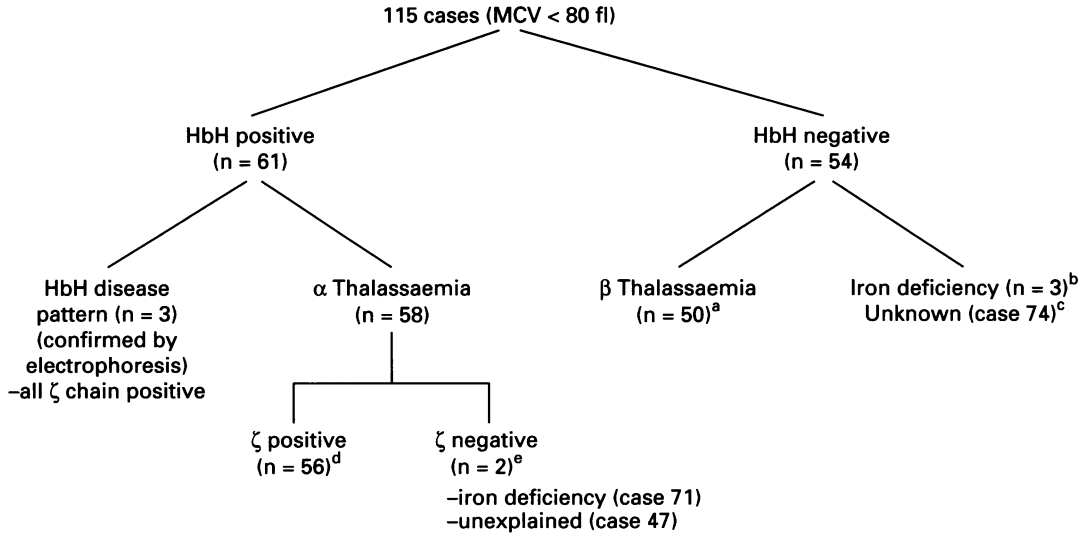


*Figure 4 Flow chart of results of the HbH inclusion test and ζ globin chain expression. [a]Five of five cases ζ chain negative; [b]two of two cases ζ chain negative; [c]ζ chain positive; [d]seven of seven cases (--/SEA) positive; [e](--/SEA) negative.*

iron deficient. The ζ chain was expressed in the patient (case 74) whose iron status was unknown and whose HbH inclusion test was negative, but not in the five cases of β thalassaemia and two cases of iron deficiency examined. As no DNA was available to resolve the α globin status of case 74, the specificity of the immunocytochemical test compared with conventional methods (after excluding case 74) for the diagnosis of α thalassaemia was 100% (7/0+7). A summary of the data is shown in fig 4.

## Discussion

The immunocytochemical detection of embryonic ζ chains is as sensitive as the HbH inclusion test in screening for α thalassaemia. Embryonic ζ chains were present in 56 (96·5%) of 58 cases of α thalassaemia 1 based on a positive HbH inclusion test. Of the two cases (47 and 74) which did not express the ζ chain, DNA analysis confirmed that the (--/SEA) deletion was absent as expected. It is possible that, in these two cases, the ζ genes were deleted together with the α gene loci, as is the case in adult carriers of deletions such as (--Fil/), (-- Thai/), and (--HW/) in South East Asia.[7 8] Case 47 had a negative discriminant function as calculated using red blood cell indexes[9] which is in keeping with the diagnosis of α thalassaemia. Other possibilities which may account for negative ζ chain expression include the -α/- α, -α/$\alpha^{CS}$α and ----/ζζαα[2] genotypes, or a "false positive" HbH inclusion test.

We have also shown that the immunocytochemical test is highly specific for the diagnosis of α thalassaemia 1. However, the ζ globin chain was also detected in a single case (case 74) for whom the HbH inclusion test was negative. As the HbF and A2 concentrations were normal and no variant haemoglobins were detected, the absence of HbH inclusions cannot be because of the presence of β thalassaemia trait or HbE, both of which are known to decrease significantly the number of HbH inclusions.[1] As no cells were stored for DNA analysis, we cannot confirm whether the (--SEA/) deletion was present using PCR.

We have shown that the immunocytochemical detection of embryonic ζ chains is a practical alternative to the HbH inclusion test in the diagnosis of α thalassaemia. As staining is positive in almost all erythrocytes, only small numbers of red cells need to be evaluated compared with the laborious screening of several fields for the detection of HbH inclusions. Detection of ζ chains using immunofluorescence can be carried out in batches and the results can be rapidly scored and assigned as definitively positive or definitively negative.

It must be emphasised that screening for embryonic ζ chains, like all laboratory tests, will need to be interpreted together with other relevant clinical and laboratory data. In a few patients with ζ-α thalassaemia the immunocytochemical test will be negative but the HbH inclusion test will be positive. Therefore, we recommend that screening for α thalassaemia be undertaken using ζ chain detection; negative cases must be confirmed using appropriate molecular analysis.

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