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International Journal of Laboratory Hematology

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LETTER TO THE EDITOR

Alpha Thalassemia Screening in Multiethnic Population in Northern Europe Using Hb Bart's Immunochromatographic Test

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Received: 3 July 2024 | **Revised:** 11 September 2024 | **Accepted:** 17 September 2024

Funding: The authors received no specific funding for this work.

Keywords: alpha thalassemia | Hemoglobin Bart's | immunochromatographic test

Dear Editors,

Alpha (α) thalassemia is one of the most common hereditary autosomal recessive disorders globally. They are often seen in malaria tropical areas as the carriers have better protection against lethal effect of this infection at young age [1]. Nevertheless, historical colonization, massive migration, and interethnic marriages for several decades may play a part in the increasing endemicity of this disorder in previously non-endemic Northern European countries and the Americas [2].

α^0-thalassemia occurs when both duplicated alpha-genes are inactivated on the same allele in majority of cases by deletions. This defect causes imbalance in alpha/beta-globin chain synthesis in which the excess of non-α chains form homotetramers: β_4 tetramers (Hb H) in adults and γ_4 tetramers (Hb Bart's) in fetus and neonates [3]. Traces of Hb H and Hb Bart's can be found in the blood of newborn carriers of α^0-thalassemia using capillary electrophoresis or high performance liquid chromatography (HPLC) but when the switch from fetal Hb F to adult Hb A occurs, the amount of homotetramers may be too small to be detected by these methods [3]. Red blood cell (RBC) stain with brilliant cresyl blue (BCB) which colors the precipitated aggregates of β_4 tetramers make inclusions visible in the erythrocytes of most carriers of α^0-thalassemia by microscopic examination [4]. This test is useful, easy to perform but labor intensive, time-consuming, and requires operator's skills to recognize these occasional to minimal intraerythrocytic inclusions.

To aid in screening for α^0-thalassemia, a sandwich type, rapid immunochromatographic test (ICT) was developed (IMED, Thailand). Monoclonal anti-Hb Bart's antibodies were generated with colloidal gold conjugate, to detect the minute amount of Hb Bart's present in α^0-thalassemia carriers and other alpha thalassemia syndromes like Hb H disease [5]. To explore the benefits of this method, to rapidly screen for potential α^0-thalassemia carriers and Hb H patients, we assessed this ICT for identification of α thalassemia syndromes in comparison with the Hb H inclusion test.

A total of 74 residuals ethylene diamine tetra acetic acid (EDTA) samples sent to our center for 9 months period, from hospital all over the Netherlands for confirmation of thalassemia and hemoglobinopathy diagnosis were tested, hence ethical approval was not required. The hematology parameters were determined by automated analyzer (Yumizen H500, Axon Lab, Germany) and Hb analyses was performed by capillary electrophoresis system (Capillarys2, Sebia, France) and HPLC (Trinity Ultra 2, Trinity Biotech, Wicklow, Ireland). Samples that were presumed having α thalassemia were selected for testing with IC strip test.

Zinc protoporphyrin (ZnPP) was measured to determine iron deficiency, as it has no or little influence in concurrent acute

TABLE 1 | Thalassemia phenotypes, genotypes of positive samples with red cell indices, ICT and Hb H inclusion body test.

Phenotype	Genotype	*N*	MCV (fl)	MCH (fmol)	ICT positive	Hb H inclusion positive
α^{+}-thalassemia trait	$-\alpha^{3.7}/\alpha\alpha$	8	67.2 ± 2.8	1.29 ± 0.07	3 (38%)	0
	α^{AATAAG} Het *HBA2*:c.*94A > G	2	68.6 ± 2.8	1.43 ± 0.07	1 (50%)	0
	$\alpha^{-(5t)}\alpha/\alpha\alpha$ Het *HBA2* c.95+2_95+6delTGAGG	1	66.2	1.40	0	0
		Alpha variant				
	$\alpha\alpha^{CD\ 119\ CCT > TCT}$ Het *HBA1* c.358C > T p.Pro120Ser	1	62.9	1.17	0	0
Homozygous α^{+} thalassemia	$-\alpha^{3.7}/-\alpha^{3.7}$	11	67.8 ± 2.9	1.33 ± 0.12	11 (100%)	0
	$\alpha^{-(5t)}\alpha/\alpha^{-(5t)}\alpha$ Hom *HBA2* c.95+2_95+6delTGAGG	5	62.1 ± 4.2	1.21 ± 0.10	4 (80%)	1 (20%)
α^{0}-thalassemia trait	$--^{SEA}/\alpha\alpha$	6	64.9 ± 6.7	1.33 ± 0.11	5 (83%)	5 (83%)
	$--^{FIL}/\alpha\alpha$	1	65.7	1.32	1 (100%)	1 (100%)
	$--^{MED\ I}/\alpha\alpha$	3	65.6 ± 4.0	1.35 ± 0.11	3 (100%)	1 (33%)
	$--^{MEDII}/\alpha\alpha$	1	67.6	1.33	0	0
	--/αα, deletion POL3RK to RGS11	2	64.8 ± 0.9	1.38 ± 0.04	2 (100%)	0
Hb H disease	$--^{SEA}/-\alpha^{3.7}$	2	60.4 ± 4.6	1.11 ± 0.06	2 (100%)	2 (100%)
α thalassemia trait	$-\alpha^{3.7}/\alpha^{Cd14TGG > CGG}$ HBA1c.43T > C p.Trp15Arg	1	65.1	1.10 ± 0.06	1 (100%)	0
	$-\alpha^{3.7}/\alpha^{-(5t)}\alpha$ HBA2c.95+2_95+6delTGAGG	1	62.5	1.28	1 (100%)	0
	Total	45			34 (76%)	10 (22%)

Note: MCH and MCV values are presented as raw data or mean ± SD.

inflammatory states [6]. Equal volume of blood and BCB 1% solution were used for Hb H inclusion test. Two blood films were prepared after incubated the solution at 37°C for 30 min. The films were microscopically examined (1000×) by two technicians and positive test was considered when one or more inclusion bodies present.

We employed the commercial kit, iLab αTHAL IC strip test kit. Lysing buffer was added into 200 μL of whole blood. The hemolysate then were tested with the test strip by immersing vertically for 2–5 min. The strips were washed with washing buffer, to remove excess hemolysate and the result was read within 10 min. A positive result indicated by presence of two bands, one in the control region and another in the capture line region. A negative band showed only one-color line in the control region [7].

All samples were tested for seven common deletions (α^+ -thalassemia: $-\alpha^{3.7}$ and $-\alpha^{4.2}$, α^0-thalassemia: Southeast Asian $--^{SEA}$, Thai $--^{THAI}$, Filipino $--^{FIL}$, MED I $--^{MED\ I}$, $-(\alpha)^{20.5}$) [8] and another two common deletions for α^0-thalassemia in our region ($--^{MED\ II}$ and $--^{DUTCH\ I}$). The samples that were negative for these aforementioned deletions were further subjected to whole *HBA* gene sequencing using universal Forward and Reverse primers and the ABI PRISM Big Dye Terminators v2.0 Cycle Sequencing Kit on an ABI PRISM 3730 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). Samples that were negative for the above tests were further tested with the MLPA technique using P140 probe for α globin gene cluster.

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for both ICT and the Hb H inclusion test based on hematological entrance criteria and compared to the molecular results obtained by GAP-PCR, MLPA, and Sanger sequencing of the HBA genes.

Among 74 subjects examined, 38 were positive for ICT and only 10 of them showed a positive Hb H inclusion test. Forty-five of the samples showed abnormalities in the α globin gene, 34 of which were positive for ICT. Table 1 summarizes the ICT and Hb H inclusion body results, the red cell indices, and molecular findings of the 45 samples.

The ICT test shows higher detection rate (76%) for α-thalassemia defects, including heterozygous α^+, homozygous α^+, and compound heterozygous α^+ and α variant, compared to the Hb H inclusion test (24%).

The sensitivity, specificity, PPV, NPV, and efficiency for ICT were 87%, 86%, 77%, 93%, and 86%, respectively, in detecting Hb H disease and α^0 thalassemia. Meanwhile, Hb H inclusion test showed low sensitivity of 60% but high specificity and PPV value of 100%, NPV value of 83%, and efficiency of 80%.

Interestingly, all samples with homozygous $-\alpha^{3.7}$ gave a positive signal in the ICT but none of them showed Hb H inclusions. 80% of homozygous $\alpha^{-(5t)}\alpha/\alpha^{-(5t)}\alpha$ were positive for the ICT and in only 20% Hb H inclusion bodies were observed. False positive and false negative results were observed in four and two samples, respectively. In the 29 samples that were negative for molecular tests for α-thalassemia, 25 had increased levels of ZnPP suggestive of iron deficiency.

Previous studies showed 98%–100% sensitivity of IC strip test in detecting α^o-thalassemia [5, 9–11]. We had two false negative samples in which one was having $--^{SEA}$ and another sample $--^{MED\ II}$ deletion. As the samples were not aged and no evidence of protein degradation were detected by CE and HPLC, we believed that clerical errors cannot be excluded. If this error was considered and the samples were excluded, the sensitivity of IC strip test can achieve 100%. Previous studies mostly tested the $--^{SEA}$ deletion, however the $--^{MED\ II}$ deletion was not tested before. Our observation in these two samples were: low haptoglobin level, indicative of hemolysis and the patient with $--^{MED\ II}$ deletion also had anemia. It is unknown if this may have caused the false negativity of these two samples.

Four samples showed false positivity with ICT without any molecular abnormality of α - globin gene. False positive results might occur due to cross-reactivity of samples with autoantibodies. Troubleshooting methods by using saline washed or RF absorbent reagent treated whole blood samples can be implemented to reduce false positivity [12].

This study showed that ICT is sensitive in detecting both heterozygous α^0 and homozygous α^+ -thalassemia. The ICT test is simple, rapid, and can be used in parallel with the abnormal hematological parameters (hypochromic microcytic indices) and normal Hb analysis.

In conclusion, our study corroborated that the ICT could potentially replace Hb H inclusion test in the screening algorithm for α-thalassemia as it has high sensitivity and high NPV for not only detecting significant α^0 carriership but also homozygous α^+. It can be applied in different targeted populations, improve the detection and pick-up rates of α-thalassemia syndromes in a developed country that has mixed ethic population. Contrariwise, it can aid in selection of α^0 carrier for further genetic testing in a country with high prevalence of α-thalassemia with limited financial resources, by incorporating this test in existing screening algorithm: hematological parameters and Hb analysis.

Author Contributions

Nik Fatma Fairuz Nik Mohd Hasan: reviewed, analyzed the study results, planned and wrote the manuscript. **Jeanet ter Huurne, Maaike Verschuren, Sharda Bhagwandien-Bisoen, and Rianne Schaap:** performed the study, result interpretation and data collection. **Sandra J. G. Arkesteijn, Linda Vijfhuizen, Hakima el Idrissi, and Tamara T. Koopmann:** verified the study results and data collection. **Cornelis L. Harteveld:** designed the study, reviewed and approved the manuscript.

Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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