Journal of Clinical Laboratory Analysis 19:22–25 (2005)

β-Thalassaemia Carrier Detection by ELISA: A Simple Screening Strategy for Developing Countries

M. Shyla Ravindran,[1] Zareen M. Patel,[1]* M. Ikram Khatkhatay,[1] and S.P. Dandekar[2]

[1]*Genetic Research Centre, National Institute for Research in Reproductive Health, Mumbai, Maharashtra, India*

[2]*Department of Biochemistry, Seth Gs Medical College & K.E.M. Hospital, Mumbai, Maharashtra, India*

The frequency of β-thalassaemia in India ranges from 3.5% to 15% in the general population and of the 100,000 children born with thalassaemia major in the world, 10,000 are in India alone. Affected children do not die immediately, but treatment by regular transfusion is costly and leads to iron overload and death. Therefore, health services in lower-economic countries can sustain patients only if the numbers can be limited. Detecting carrier couples by simple blood test can prevent thalassaemia and at-risk couples can be identified and informed of their genetic risk before having children. A prevention programme including population screening, counselling, and prenatal diagnosis will markedly reduce the birth prevalence of affected individuals. Hemoglobin A_2 (HbA_2) measurement in human hemolysates has great significance, since its level can indicate β-thalassaemia carrier status in otherwise healthy individuals. We have developed a rapid, simple, and inexpensive enzyme linked immunosorbent assay (ELISA) for the quantitation of HbA_2, which can be used in carrier screening programmes in developing countries like India. In a limited trial for β-thalassaemia carrier screening, the results obtained with ELISAs were compared with those obtained with the microcolumn chromatography method ($r = 0.89$). J. Clin. Lab. Anal. 19:22–25, 2005.

Key words: hemoglobin A_2; enzyme linked immunosorbent assay; India, control of thalassaemia; simple method

INTRODUCTION

The enzyme linked immunosorbent assays (ELISAs) are generally regarded as having the same degree of sensitivity as the corresponding radioimmunoassays, but have the additional advantage of utilizing more stable reagents and not requiring radioactive preparations and specialized counters (1). In normal individuals, the mean proportion of hemoglobin A_2 (HbA_2) is 2.5% of total hemoglobin, whereas that for β-thalassaemia heterozygotes it is high and varies from 3.7% to 7.0%. This abnormally high concentration of HbA_2 is a generally accepted principal diagnostic feature. HbA_2 is generally estimated by ion exchange chromatography or cellulose acetate electrophoresis. The estimation of HbA_2 by ion exchange chromatography depends on the interchange of charged groups on ion-exchange resin (diethyl aminoethyl cellulose: DEAE-cellulose) with charged groups on the hemoglobin molecule. In this method HbA_2 can be separated on a micro column of resin and quantitated by spectrophotometry (2). In cellulose acetate electrophoresis, at pH 8.9 HbA_2 is separated from HbA and eluted into buffer. The absorbance of HbA_2 is measured and compared with that of the remaining hemoglobin to calculate a percentage. This method is unsuitable in presence of hemoglobins with electrophoretic mobility similar to that of HbA_2. The results obtained from these methods are not reproducible and techniques require well-trained personnel.

Making use of the difference in structures of β- and δ-chains of HbA and HbA_2, respectively, to raise antisera specific for the δ-chain and employ it for an immunological assay (ELISA) to quantitate HbA_2 is the basic concept of this study. This is a novel approach and may

Grant sponsor: Indian Council of Medical Research; Grant number: NIRRH/22/2004.

*Correspondence to: Z. M. Patel, Genetic Research Centre, National Institute for Research in Reproductive Health, Jehangir Merwanji Street, Parel Mumbai 400012, Maharashtra, India.
E-mail: zmpatel@hotmail.com; grcnirrh@rediffmail.com

Received 30 July 2004; Accepted 15 October 2004
DOI 10.1002/jcla.20050
Published online in Wiley InterScience (www.interscience.wiley.com).

be the first initiative of its kind in our country. This technique requires the incorporation of nonspecific antiserum capable of specifically reacting only with HbA_2, i.e., with the δ-chain of HbA_2. The ELISA technique is more sensitive and specific than the biochemical methods used currently (5). The ELISA can be employed either in conjunction with routine methods of β-thalassaemia screening (e.g., blood cell indices, naked eye single tube red cell osmotic fragility test (NESTROFT), etc.) or can be used alone to screen populations. Qualitative or quantitative ELISAs may be used to suit our requirements, eliminating the use of expensive and time-consuming techniques.

In developed countries, self-contained and well-simplified ion-exchange chromatography kits are available. Even though they are very expensive, they are routinely used in these countries. Perhaps this might have prevented the development of ELISA-based diagnostic kits for screening of thalassaemia. However, immunological assays for various hemoglobins have been successfully developed and used in the United States. A very sensitive assay could be significant in detecting small amounts of HbA_2in hemolysates and could replace the more expensive and tedious methods, especially in large screening programmes in our country.

MATERIALS AND METHODS

For the present study, 350 subjects were analyzed for their β-thalassaemia status. The first group of subjects were 150 women attending the antenatal outpatient department of a maternity hospital in Mumbai. The second group studied was 200 volunteers attending various outpatient departments of a hospital.

In the first group, samples were analysed for their β-thalassaemia status by the routine methodology described elsewhere (3). All the patients were enrolled after filling out a form that included details such as name, age, address, community, consanguinity, week of their gestation, etc. The subjects of the second group were analyzed for their β-thalassaemia status only by ELISA, followed by a column chromatography.

The blood of each patient (2 mL) was collected in anticoagulant and subjected to NESTROFT, estimation of hemoglobin, and red cell indices. Hemolysates were prepared and subjected to cellulose acetate electrophoresis at alkaline pH. All the hemolysates were then subjected to HbA_2 estimation by microcolumn chromatography as well as ELISA.

The following criteria were used to identify β-thalassaemia traits. NESTROFT positivity, positive family history, decreased red cell indices mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH), anaemia, and HbA_2 levels more than 3.5%. The blood of each patient (1 mL) was collected in ethylene diamine tetra acetic acid (EDTA) vials. Hemolysates were prepared and ELISA was carried out to quantitate HbA_2. The protocol was essentially similar to that described by Graver (5) with suitable modifications required for the enzyme HRP. The wells of polystyrene microtitre plates (Nunc modules) were adsorbed with 200 μL of adsorption buffer (100 mM/L bicarbonate buffer, pH 9.6.) containing 125 μg of HbA_2 per milliliter of buffer. After overnight incubation at 4°C, plates were washed three times with 100 mM/L phosphate buffer, pH 7.2, containing 0.05% Tween-20 (PBST). Unoccupied sites were blocked by adding 200 μL of 1% w/v bovine serum albumin, in 100 mMol/L PBS, pH 7.2, to each well and incubated at room temperature for 1 hr, followed by three washes in PBST.

For quantitative analysis, dose response curves were developed by preparing varying concentrations of HbA_2 in HbA, ranging from 0–10% of HbA_2 in HbA, and the final concentrations to 50 μg of total Hb/mL. This was achieved by preparing a stock solution of HbA (500 μg/mL) and HbA_2 (50 μg/mL) and combining them in varying proportions to get the required percentages, with final protein concentration constant at 50 μg/mL. The sample hemolysates were also diluted accordingly, to 50 μg/mL with PBST. To each well of a microtitration plate, 150 μL of anti-HbA_2 diluted 1:1,000 in PBST was dispensed. At the end of 90 min incubation at 37°C, the wells were rinsed three times with PBST.

Then, 200 μL of 1:5,000 diluted antirabbit gama globulin-horseradish peroxidase (ARGG-HRP) conjugate was dispensed. After 1 hr at 37°C, the wells were washed three times with PBST, followed by the addition of 200 μL of ortho-phenylenediamine (OPD) in citrate buffer containing 0.5μL of H_2O_2. The reaction was terminated by the addition of 4N H_2SO_4 after 20 min. Absorbance was measured at 490 nm on an ELISA reader (Biotech, Winooski, VT). All analyses were performed in duplicate and results were expressed as the mean of these determinations. HbA_2 was also estimated by microcolumn chromatography (Bio-Rad, Milano, Paris) as per the instructions of the manufacturer. The results were then compared.

RESULTS

The mean HbA_2 value for a normal individual was 2.373% and the value ranged from 1–3.5% with a standard deviation (SD) of 0.49%. The 95% confidence interval (CI) was 2.32–2.42% (mean $2.37 \pm$SD 0.052). The mean HbA_2 value for a β-thalassaemia carrier was 4.163% and the value ranged from 3.6–4.8% with an SD of 0.365%. The 95% CI was 3.98–4.34% (mean $4.16 \pm$SD 0.179).

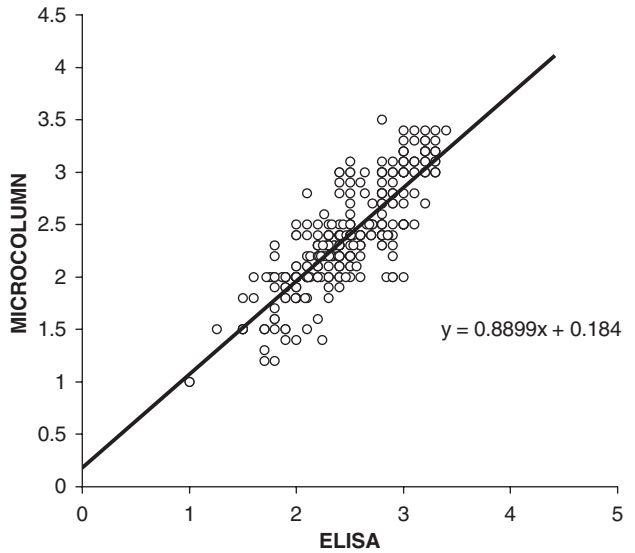

Fig. 1. Comparison of HbA_2 values as estimated by ELISA with microcolumn chromatography in normal individuals.

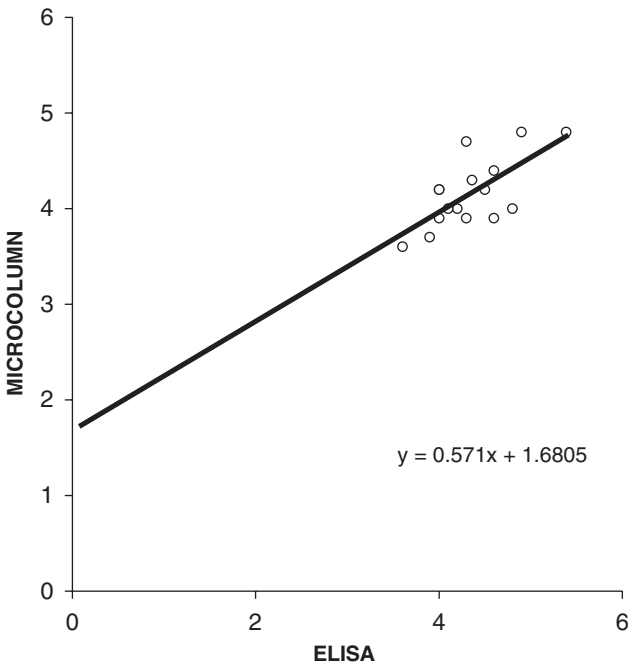

Fig. 2. Comparison of HbA_2 values as estimated by ELISA with microcolumn chromatography in β-Thalassaemia carriers.

The two cases in which HbA_2 values were >10 and electrophoresis showed the presence of hemoglobin E, the HbA_2 values were 2% and 1.9%. This finding shows the more specific nature of the ELISA, i.e., the monospecific nature of the assay to HbA_2. This could be a negative aspect of the ELISA, by missing out on the presence of some variant hemoglobin that may be present in the sample.

HbA_2 was estimated by ELISA and microcolumn chromatography in 350 samples enrolled in this study. The values obtained by both the methods compared well with a correlation coefficient of 0.89 ($n = 350$, $P < 0.001$) (Figs. 1 and 2) (4).

The ELISA values were on an average 3.9% lower than the values obtained by microcolumn chromatography.

DISCUSSION

Combined data from different assays provides quick identification hemoglobin variants. Although the ultimate identification of an abnormal hemoglobin (diagnosis of a disorder) is confirmed only by structural analysis or by gene mapping. These procedures are still beyond the capabilities of clinical laboratories in our country.

As seen in Table 1, the other methodologies for carrier detection have the disadvantage of having very low positive predictive value. When microcolumn chromatography was used, the positive predictive value rose to 100% when HbA_2values $>10\%$ are not included in the positive category. But when HbA_2 values $>10\%$ are included in the positive group, the predictive value dropped to 71.4%.

Laboratory diagnosis of the β-thalassaemia trait primarily depends up on the quantitation of HbA_2 in adult hemolysates. For screening purposes, the most widely accepted technique is microcolumn chromatography (5). The immunological methods are not only much cheaper than these microcolumns, but more specific, sensitive, and reproducible.

Screening and antenatal diagnosis programmes have been applied widely in Mediterranean populations. Their success has been based on the excellence of public education programmes about the disease, followed by the development of effective screening regimens and facilities for antenatal detection. For religious, cultural, organizational, and economic reasons, programmes of this type may be much more difficult to establish in the large mainland populations of the Indian subcontinent and Southeast Asia (6). Clearly it will be for individual governments and communities to decide how far they want to go down this road but, as a start, education programmes should be established, backed up with facilities for carrier screening on a voluntary basis. This type of approach is the bedrock on which further population control programmes can be built, if and when individual countries wish to pursue them.

Although the carrier status for the important hemoglobin disorders can be identified by simple laboratory techniques, the availability of a cheaper alternative can be a big boost to screening programmes in developing

TABLE 1. Summary of assay characteristics of different methodologist for β − Thalassaemia carriers (n = 150)

	Nestroft	Cell indices	Electrophoresis	Column chromatography (>10% HbA2 considered)	ELISA
Sensitivity	100%	100%	100%	100%	100%
Specificity	88.9%	86.2%	91%	98.6%	99.3%
Accuracy	89.3%	86.6%	91.3%	98.7%	99.3%
−Ve predictive value	100%	100%	100%	100%	100%
+Ve predictive value	23.8%	20%	27.8%	71.4%	83.3%

countries like India. The ELISA standardized in this study fully conforms to the above simple, economical, and specific criteria, making it a suitable alternative for the future. The establishment of some central reference laboratories for DNA diagnostics is very essential in our country, where the frequency of disorders is very high.

The ELISA developed successfully in this study has great utility in control of thalassaemia in developing countries like India. This ELISA is simple, accurate, precise, inexpensive, and several samples can be processed simultaneously, with ease, making this system a suitable candidate for transforming into a user-friendly kit. Conceivably, this technique could be adapted for use in mass screening programmes for β-thalassaemia carriers.

CONCLUSION

Immunoidentification (via ELISA), with its high specificity, sensitivity, accuracy, and economy combined with qualitative electrophoresis, appears to be ideal for future routine identification and quantitation of hemoglobins of clinical significance in India.

ACKNOWLEDGMENTS

We thank Dr. C. P. Puri, Director, National Institute for Research in Reproductive Health (NIRRH) and Dr. Moodbidri for the support given to this study.

REFERENCES

1. Engvall E, Perlmann P. Enzyme linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme labelled anti-immunoglobulin in antigen coated tubes. J Immunol 1972;109:129–135.
2. Huisman THJ, Dozy AM. Studies on the heterogeneity of hemoglobin. J Chromatogr 1965;19:160–169.
3. Maheswari M, Arora S, Kabra M, Menon PSN. Carrier screening and prenatal diagnosis of β-thalassaemia. Indian Pediatr 1999;36:1119–1125.
4. Menon SR, Khatkhatay MI, Dandekar SP, Patel ZM. Development and validation of an ELISA for hemoglobin-A_2: a novel method for β-thalassaemia screening in developing countries. J Immunoassay Immunochem 2004;25:135–146.
5. Grenet HE, Graver FA. Identification and quantitation of sickle cell hemoglobin with an enzyme-linked immunosorbent assay (ELISA). J Clin Lab Med 1980;96:597–605.
6. Weatherall DJ, Clegg JB. Inherited haemoglobin disorders: an increasing global health problem. Bull World Health Organ 2001;79:704–712.