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Reference intervals for HbA_2 and HbF and cut-off value of HbA_2 for β-thalassemia carrier screening in a Guizhou population of reproductive age

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ABSTRACT

Objective: The aims of this study were to establish the reference intervals for HbA2 and HbF in a Guizhou population of reproductive age, and to determine the cut-off value of HbA_2 for β-thalassemia carrier screening.
Methods: Hemoglobin analysis was performed on 832 individuals without hypochromic microcytic anemia to calculate the reference intervals for HbA_2 and HbF. Three hundred and ninety one β-thalassemia carriers and non β-thalassemia individuals were analyzed for their HbA_2 levels followed by detecting β-globin gene mutations, then cut-off value of HbA_2 for β-thalassemia carrier screening was determined using ROC curve analysis.
Results: The reference interval for HbA_2 in overall normal individuals was 2.3%–3.1%, and reference intervals for HbF in normal males and females (including normal females and pregnant women) were 0–0.5% and 0–1.0% respectively. The cut-off values of HbA_2 for β-thalassemia carrier screening in males, non-pregnant women, pregnant women and the overall set were 4.40%, 3.75%, 3.70% and 3.95% respectively.
Conclusion: Gender and pregnancy status had no obvious influence on reference interval for HbA_2. The HbF level was higher in females than in males, but pregnancy status had no obvious influence on HbF level. Cut-off value of HbA_2 for β-thalassemia carrier screening was obviously affected by gender but not by pregnancy status.

1. Introduction

β-thalassemia is one of the most common single gene genetic disorders worldwide, characterized by a hypochromic microcytic anemia due to point mutations as well as small fragment insertions or deletions in β-globin gene. It is estimated that about 1.0% to 5.0% of the world's population carry β-thalassemia genes [1]. The carrier frequency of β-thalassemia in Guizhou Province located in Southwest China is relatively high, ranging from 2.66% to 7.85% among different ethnic groups [2–4]. When both parents are carriers of β-thalassemia, they have a 25% (1 in 4) chance with each pregnancy of having a child with β-thalassemia major or intermedia. Therefore, carrier screening for β-thalassemia in population at reproductive age is important for β-thalassemia control. Because α- and β-thalassemia carriers are both characterized by microcytosis and hypochromia, mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) are the most common two major clinical indicators for thalassemia carrier screening [5,6]. In addition, β-thalassemia carriers markedly exhibit elevated hemoglobin A_2 (HbA_2), and probably increased fetal hemoglobin (HbF) [7]. Therefore, elevated HbA_2 is the most important blood parameter for the identification of β-thalassemia carriers and plays a key role in screening programs for β-thalassemia. The normal reference intervals for HbA_2 and HbF were 2.5% -3.5% and less than1.0% respectively in several studies based on Chinese population, and the conventional cut-off value of HbA_2 was > 3.5% for β-thalassemia carrier screening. However, as the normal reference intervals for HbA_2 and HbF can be affected by gender, pregnancy, disease status, race, geographic region, and detection methods [8–11],and the cut-off value of HbA_2 could be varied in different population as well as in different laboratories. Therefore, it is necessary to reassess the reference intervals for HbA_2 and HbF as well as the cut-off value of HbA_2 for β-thalassemia carrier screening in different populations and/or laboratories.

2. Methods

2.1. Subjects

Between January 2016 and June 2017, 1223 individuals of

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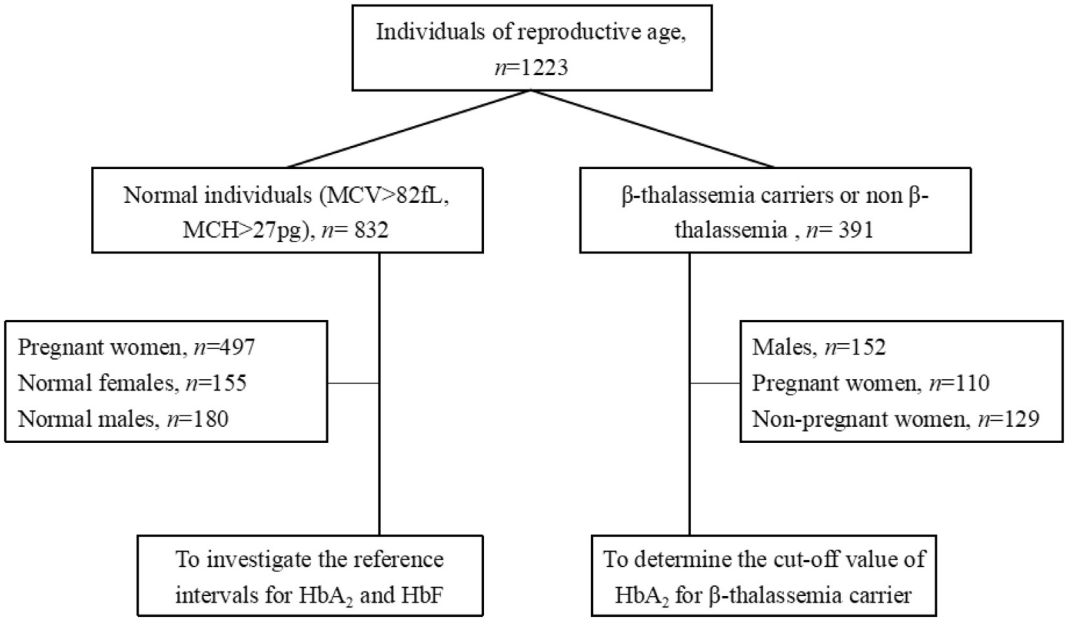

Fig. 1. Flow chart for participating individuals.
A: HbF data distributions in normal groups; B: HbA_2 data distributions in normal groups; C: HbA_2 data distributions in non β-thalassemia groups; D: HbA_2 data distributions in β-thalassemia carrier groups.

Table 1
Number of cases and age of each subjects group.

	Number of cases	*Mean* ± *SD* of age (min~max)
Subjects for reference intervals	832	31.0 ± 5.7(20–45)
Pregnant women	497	28.0 ± 3.7(20–43)
Normal females	155	32.4 ± 6.9(20–45)
Normal males	180	32.5 ± 6.9(20–45)
Subjects for cut-off values of HbA_2	391	29.9 ± 6.0(20–45)
Males	152	31.7 ± 6.1(20–45)
Pregnant women	110	28.0 ± 5.0(20–40)
Non-pregnant women	129	30.1 ± 6.3(20–45)

reproductive age from 18 to 45 years old from Guizhou Province referred to the Guizhou Provincial People's hospital for prenatal screening of thalassemia, β-thalassemia genetic testing, or healthy examination were enrolled. Of the 1223 individuals, 832 normal individuals without hypochromic microcytic anemia (MCV > 82 fL, MCH > 27 pg) and other disorders with high levels of HbA_2 or HbF, such as hematopoietic malignancies, myelodysplastic syndrome, were used to investigate the reference intervals for HbA_2 and HbF. These subjects were divided into three groups: normal pregnant women (12–20 gestational weeks), normal females and normal males. The remaining 391 individuals, including 96 β-thalassemia carriers with hypochromic microcytic anemia (MCV < 82 fL, MCH < 27 pg) and elevated level of HbA_2 and 295 non β-thalassemia individuals confirmed by genetic testing, were applied to determine the cut-off value of HbA_2 for β-thalassemia carrier screening, which also divided into three groups: males, pregnant women (12–20 gestational weeks) and non-pregnant women (Fig. 1). The number of cases and age of each group was listed in Table 1. This study was approved by the Ethics Review Committee of the Guizhou Provincial People's Hospital. All subjects gave written informed consent prior to their participation in the study.

2.2. Sample collection and processing

Two-milliliter of venous blood was collected from each participant by venipuncture into an EDTA-Na_2 anticoagulant vacutainer tube. Hemoglobin capillary electrophoresis was performed on a CAPILLARYS 2 instrument (Sebia, France) within 12 h after blood samples were collected. Seventeen common β-thalassemia mutations in Chinese population, namely, codons 41/42 (–CTTT), IVS-II-654 (C → T), codon 17(A → T), −28 (A → G), codons 71/72 (+A), βE (codon 26 G → A), codon 43(G → T), −29 (A → G), codons 27/28 (+C), IVS-I-1 (G → T), IVS-I-5 (G → C), codon 31 (−C), −30 (T → C), −32 (C → A), codons 14/15(+G), initiation codon (ATG → AGG) and Cap (-AAAC) were detected by PCR-reverse dot blot (PCR-RDB) technique.

2.3. Statistical analysis

The data of HbA_2 and HbF of each group were firstly tested for normality by using Shapiro-Wilk tests, then a box-and-whisker plots was used to show the data distribution. Reference intervals for HbA_2 and HbF were determined according to the Clinical and Laboratory Standards Institute (CLSI) C28-A3c guideline [12]. Briefly, outliers were removed with the Dixon's rule; Harris and Boyd test was used to determine whether the reference intervals need to be partitioned; The reference interval of each group was calculated using the non-parametric method to calculate the lower and higher reference limits, respectively. For each reference interval, 90% confidence intervals (CI) were calculated for either end.

The receiver operating characteristic (ROC) curves of HbA_2 for each cut-off value determination group (males, pregnant women, non-pregnant women) were drawn, then ROC curves were used to determine optimal cut-off values of HbA_2 for β-thalassemia carrier screening by calculating the Youden index. All the data were analyzed by SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Data distribution

Normality and homogeneity test for variance found that HbA_2 data were normally distributed with the same variance in each group. However, the majority of subjects have a value of HbF under the

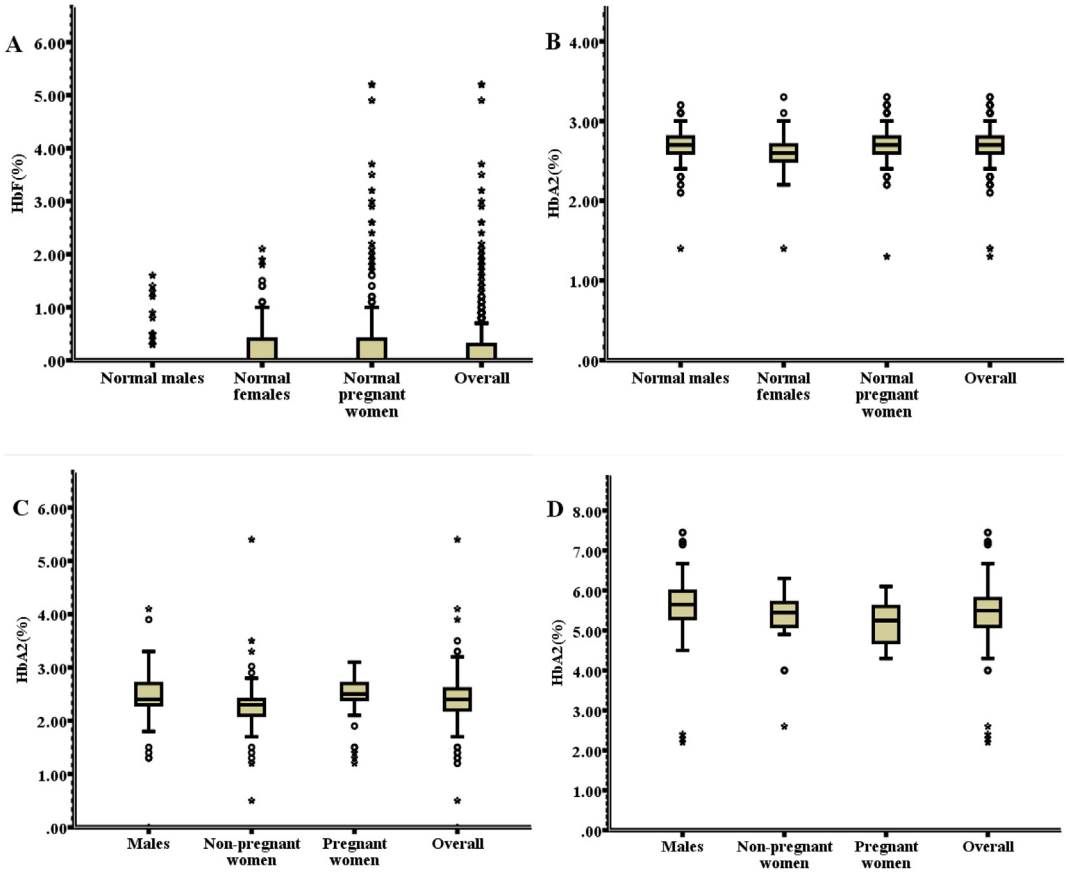

Fig. 2. Box-and-whisker plots of HbA_2 and HbF.
A: males group; B: non-pregnant women group; C: pregnant women group; D: overall set.

detection limit, with the proportions of subjects had HbF values of 0 in normal males, normal femals and pregnant women were 91.7% (165/180), 69.0% (107/155) and 70.2% (349/497), respectively. As such, the data distribution of HbF was skewed even after the log transformation was applied. The Box-and-Whisker plots of HbA_2 and HbF of all groups were shown in Fig. 2.

In normal group, the overall mean value of HbA_2 was (2.70 ± 0.21) %, and the median and interquartile range of HbF were 0 and 0.3% respectively. The mean value of HbA_2 in normal females (2.63 ± 0.21) % was significantly lower than that in normal males (2.70 ± 0.23) % and normal pregnant women (2.70 ± 0.19) % ($P < 0.01$). All the medians of HbF in the three groups were 0. However, the interquartile range of HbF in normal male still was 0, while the interquartile ranges of HbF in normal females and normal pregnant women were both 0.4% (Supplement Table 1). With non-parametric test, the distributions of HbF between normal males, normal females and pregnant women were significantly different ($P < 0.01$).

3.2. Reference intervals for HbA_2 and HbF

According to Harris and Boyd test, we calculated Z values with the standard deviation and a modified *Z*-statistic. It was not necessary to partition the reference interval for HbA_2, while that of HbF required gender grouping (Supplement Table 2). Median, 95% percentile and 90% CI of reference intervals for HbA_2 and HbF in each group are shown in Table 2. Since the results of hemoglobin test with Sebia electrophoresis apparatus only reserve one decimal place, the reference interval for HbA_2 in overall normal individuals obtained from Table 2 was 2.3%–3.1%, and the reference intervals for HbF in normal males and females (including normal females and pregnant women) were 0–0.5% and 0–1.0% respectively.

3.3. Cut-off value of HbA_2 forβ-thalassemia carrier screening

Taking Genetic testing as the gold standard, three groups of subjects were subdivided into β-thalassemia carriers (also called β-thalassemia minor) and non β-thalassemia. The most common mutation types in this study subjects were codons 41/42 (–CTTT), IVS-II-654 (C → T), codon 17(A → T), −28 (A → G). There was no obvious relationship between these mutations and the levels of HbA_2 and HbF (Supplement Table 3). In the three groups of males, non-pregnant women and pregnant women, the HbA_2 level in each β-thalassemia carrier subgroup was higher, with a statistically significant difference compared to the non β-thalassemia subgroup ($P < 0.001$) (Supplement Table 4).

ROC curves of overall set and each group are shown in Fig. 3. The cut-off values of HbA_2 for β-thalassemia carrier screening in males, non-pregnant women, pregnant women and the overall set were 4.40%, 3.75%, 3.70% and 3.95% respectively, with accuracies all above 98.0% (Table 3). On the basis of cut-off value of 3.95% in overall set to calculate, the sensitivity increased from 92% to 94% and positive predictive value decreased from100% to 97.9% in males group, specificity and accuracy were also slightly decreased, nevertheless the diagnostic indicators in non-pregnant women and pregnant women group were almost unchanged (Table 4).

4. Discussion

Although HbF and HbA_2 are two important indicators for β-thalassemia carrier screening, there were no unified reference intervals for these two indicators in different populations and cut-off value of HbA_2 for β-thalassemian carrier screening, because of the test results can be influenced by a variety of factors, e.g. gender, race, region, pregnancy status and detection methods. There were few extensive studies on

Table 2
Range, median, IQR, 95% percentile and 90% CI of reference intervals for HbA_2 and HbF (%).

Parameters	Group	N	Range (min-max)	Median (IQR)	RI (95% percentile)	Lower limit 90%CI	Upper limit 90%CI
HbA_2 (%)	NM + NF + PW	832	1.30–3.30	2.70 (2.60–2.80)	2.29–3.11	2.29 (2.10–2.30)	3.11 (3.10–3.20)
HbF (%)	NM	180	0.00–1.60	0.00 (0.00–0.00)	0.00–0.50	0.00 (0.00–0.00)	0.50 (0.03–0.90)
	NF + PW	652	0.00–7.10	0.00 (0.00–0.40)	0.00–1.04	0.00 (0.00–0.00)	1.04 (0.90–1.40)

NM, normal males; NF, normal females; PW, pregnant women; IQR, interquartile range; RI, reference interval; CI, confidence interval.

reference intervals for HbA_2 and HbF as well as the cut-off value of HbA_2 for β-thalassemian carrier screening in Chinese population. Most laboratories adopt Manufacturers' recommended reference intervals for HbA_2 and HbF, for example Sebia recommended that the normal reference intervals for HbA_2 and HbF were 2.5%–3.5% and < 1.0% respectively. In order to perform more accurate β-thalassemian screening using of HbA_2 and HbF values, it is necessary to establish the reference intervals and cut-off values based on local population.

In this study, a Guiyang population of reproductive age were enrolled to investigate the reference intervals for HbA_2 and HbF. On the whole, the reference interval for HbA_2 was 2.3% – 3.1%, which is close to those of Nanning, China and Malaysia population (both were 2.3%–3.3%) established by the same CE method [13,14], but slightly lower than that recommended by Sebia (2.5%–3.5%), which were derived from Western population. Therefore, reference interval for HbA_2 derived from different regions and populations is discrepant. As for the HbF level, there was no obvious difference between regions and populations, while the differences are mostly depending on detection methods. According to the previous reports, the reference interval for HbF determined by CE was usually < 1.0%, while by high performance liquid chromatography (HPLC) it was usually < 2.0% [16]. This could be due to the presence of glycated hemoglobin (hemoglobin A1c, HbA1c) fractions that could overlap with HbF in HPLC analysis resulting elevation of HbF level [16].

In the present study, we also explored the influence of gender and pregnancy status on reference intervals for HbA_2 and HbF. Although the mean values of HbA_2 in normal males and normal pregnant women (both were 2.70%) were significantly higher than that in normal female (2.63%), partitioning was not needed when reference interval for HbA_2 was determined according to Harris and Boyd test. Therefore, we can use the same reference interval for HbA_2 despite of gender and pregnancy status. Our results also showed that males and females had

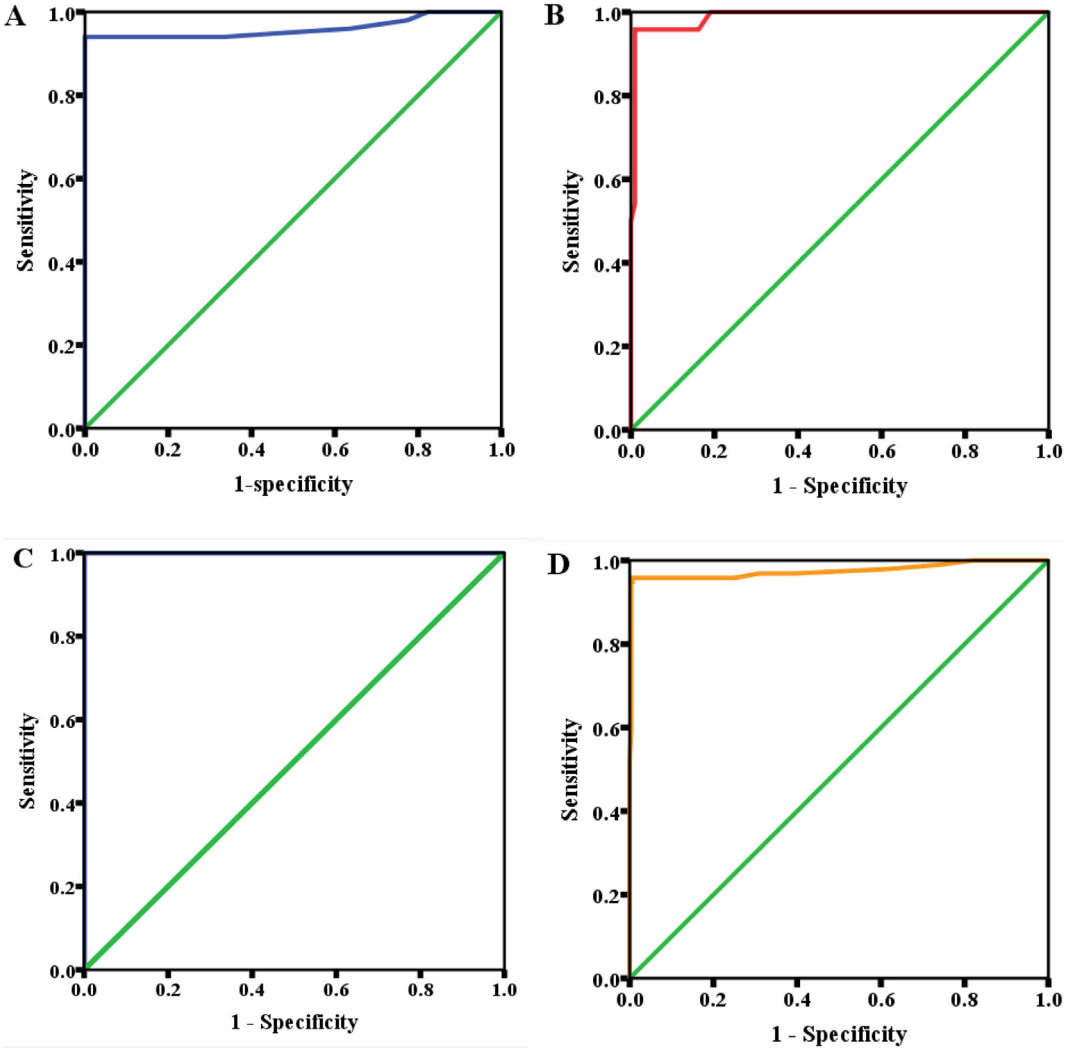

Fig. 3. The ROC for HbA_2 of each group.

Table 3
Cut-off value of HbA_2 and diagnostic indicators in each group (%).

Group	Cut-off value	AUC (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Males	4.40	0.94	92	100	100	97.1	98.0
Non-pregnant women	3.75	0.99	95.8	99.0	95.8	99.1	98.5
Pregnant women	3.70	1.00	100	100	100	100	100
Overall	3.95	0.97	94.8	99.3	97.9	98.7	98.5

PPV, positive predictive value; NPV, negative predictive value.

Table 4
Diagnostic indicators calculated based on the overall HbA_2 cut-off value of 3.95%.

Group	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Males	94.0	99.0	97.9	97.1	97.4
Non-pregnant women	95.8	99.0	95.8	99.0	98.4
Pregnant women	100	100	100	100	100

PPV: positive predictive value, NPV: negative predictive value.

different reference intervals for HbF, with upper limit were 0.5% and 1.0% respectively, indicating that the level of HbF was higher in females than that in males. But pregnancy status (normal females and normal pregnant women) had no obvious influence on HbF level, and we can use the same reference interval for HbF in both pregnant and non-pregnant women.

As noted in previous reports, HbA_2 was a highly sensitive and specific index for β-thalassemia carrier screening. However, the cut-off value of HbA_2 for β-thalassemian carrier screening was varied according to different reports. In this study, a total of 391 individuals of reproductive age in Guiyang region whose β-globin gene genotype were determined were enrolled for β-thalassemia carrier screening. The results showed that the overall cut-off value of HbA_2 was 3.95% with rather high sensitivity, specificity and accuracy of 94.8%, 99.3% and 98.5% respectively. The cut-off value of HbA_2 in this study was higher than most reports based on other Chinese populations, but almost consistent with those reported by Ceng et al. and Huo et al. [17,18], and lower than that reported by Zhang et al. (4.35%) [19]. The reason may be related to different regional populations and different testing methods, which can influence the testing results of HbA_2. In addition, our results showed that the cut-off value of HbA_2 in male (4.40%) was significantly higher than those in non-pregnant women (3.75%) and pregnant women (3.70%), indicating cut-off value of HbA_2 obviously affected by gender but not by pregnancy status. With the overall cut-off value (3.95%) was adopted, the accuracy of β-thalassemia carrier screening in males was slightly decreased, while diagnostic indicators of two female groups were almost unchanged. Just like many other laboratories, we used the same traditional HbA_2 cut-off value of 3.5% for all individuals. In order to reduce the false positive rate, it is recommended that the cut-off value of HbA_2 for β-thalassemian carrier screening is 4.40% for males and 3.70% for females which can improve the accuracy of β-thalassemian screening in Guizhou population of reproductive age. However, we need to implement the gender specific cut-off values in local population for a period of time to validate their efficiency on β-thalassemia carrier screening.

In conclusion, each laboratory should establish its own reference intervals for HbA_2 and HbF based on different detection methods, and determine the cut-off value of HbA_2 for β-thalassemian carrier screening, also considered various factors such as gender, pregnancy status to improve the accuracy of screening results.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2018.11.007.

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