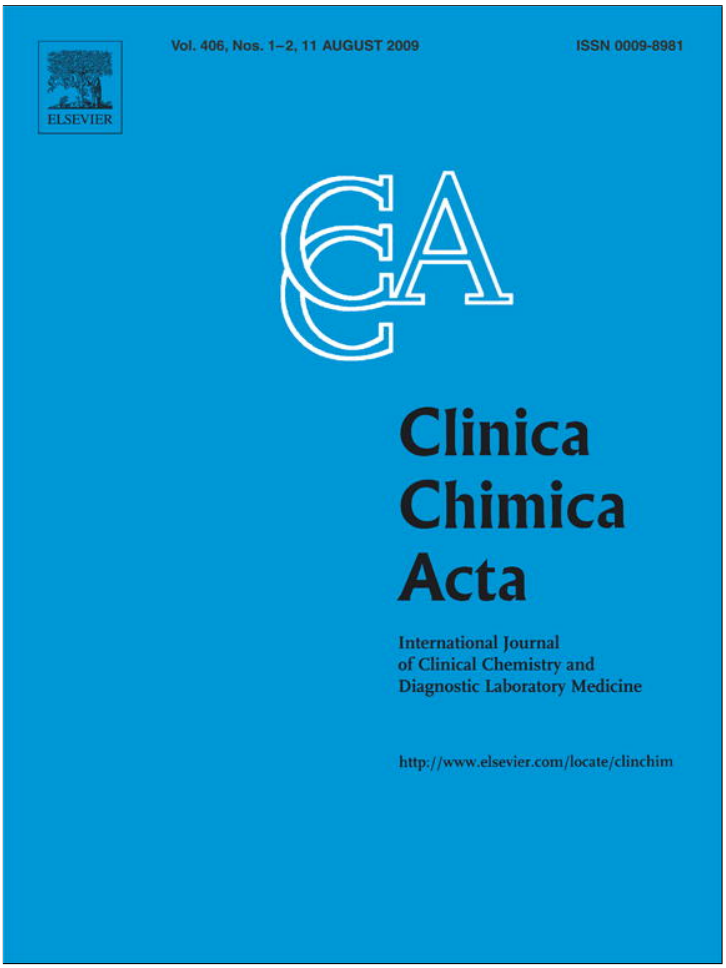

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Reporting variant hemoglobins discovered during hemoglobin A_{1c} analysis — Common practices in clinical laboratories

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ABSTRACT

Background: Patients with variant hemoglobins may receive inaccurate results by some HbA_{1c} methods. We examined reporting practices of clinical laboratories with respect to variant hemoglobins and limitations of methodology.
Methods: A survey of reporting practices was published in *LabMedicine*, and circulated to directors of Clinical Laboratory Sciences programs. Websites of reference laboratories were reviewed.
Results: One hundred thirty-five laboratories from 42 US states responded. 61.5% of those laboratories report only HbA_{1c} value and reference interval; 5% of laboratories include methodology. 51% of laboratories use IE-HPLC, 47% use immunoassay and 2% use boronate affinity chromatography. Of laboratories using IE-HPLC, 39% routinely report the presence of hemoglobin variants, and 10% report variants only if they cause interference with the test. Of laboratories using immunoassay, only one appends the disclaimer that elevated HbF interferes with test results. All of the major reference laboratories report methodology on their websites; only 2 can detect hemoglobin variants. Six out of 7 reference laboratories state limitations of methodology on their websites.
Conclusions: There is no standardized reporting format for HbA_{1c} that includes methodology, test limitations or notification of variant hemoglobins. An algorithm for detection and reporting of variant hemoglobins and test methodology is proposed based on best practices.

1. Introduction

Since the first efforts to standardize Glycohemoglobin measurements were reported in 1992 [1], the accuracy and precision of Hemoglobin A_{1c} (HbA_{1c}) has improved, and its utilization has increased. The standard of care for a patient with diabetes mellitus includes HbA_{1c} testing 2–4 times per year [2]. The therapeutic goal of HbA_{1c} is <7%, based on studies that showed increased incidence of microvascular sequelae in patients above this level [3,4]. The goal is appropriate for patients with either type 1 or type 2 diabetes [2]. A change of 1% HbA_{1c} is equivalent to a change of ~29 mg/dl (1.6 mmol/l) in the average blood glucose [5].

There is a wide variety of sophisticated instrumentation and small point-of-care analyzers for the measurement of HbA_{1c}. Results from the CAP 2008 GH2-B survey indicated that immunoassay and ion-exchange high performance liquid chromatography (IE-HPLC) were the major methods used in the US, followed by boronate affinity chromatography [6]. Over 99% of the laboratories reporting in the CAP survey used a method that is certified by the National Glycohemoglobin Standardization Program (NGSP). The goal of the NGSP is that HbA_{1c} results from one laboratory using a NGSP certified method should be interchangeable with another laboratory using the same method or another NGSP certified method. For patients with variant hemoglobins and/or elevated hemoglobin F, this may not be the case.

HbA_{1c} is measured relative to total hemoglobin, i.e. $HbA_{1c}/(HbA_{1c}+HbA)$. Over 1000 variant hemoglobins have been identified [7]. These non-A hemoglobins may interfere with immunoassay if their glycated forms are not recognized with the same affinity as glycated hemoglobin A by the reagent antibody. Hemoglobin F interferes with immunoassay HbA_{1c} methods because glycated HbF cannot react with the reagent antibody, yet the total hemoglobin includes HbF, i.e. $HbA_{1c}/(HbA_{1c}+HbA+HbF)$. Elevated HbF therefore lowers the HbA_{1c} when it is in sufficient concentration. Hb variants can interfere with IE-HPLC if they co-elute with HbA_0 or HbA_{1c}, or if the Hb variant peaks have retention times that are very close to one of these peaks [8,9]. Manufacturers of immunoassays and IE-HPLC strive to address these issues, and many methods deliver accurate results in the presence of one or more of the major Hb variants [10]. HbF is quantitated as a separate peak by IE-HPLC, and its concentration is the deciding factor as to whether the HbA_{1c} is considered reportable. The HbF cutoff is method dependent; some methods are accurate up to 30% HbF, some are accurate only to 5% [10,11]. Boronate affinity chromatography results are comparable to the IFCC reference method, mass spectrometry, for samples with HbS and HbC trait [12], and is used as a comparison method for HbA_{1c} in the presence of HbE and HbD [13,14], but shows a negative bias with

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increased HbF [11]. Hemoglobinopathies may also affect results if they cause decreased red cell survival or do not glycate at the same rate as HbA [8].

The presence of non-A hemoglobin may cause a spurious increase or decrease in the HbA_{1c} result, depending on the methodology and the properties of the hemoglobin [11,13,14]. In the US, the patients most affected have HbAS, AC, AE and elevated HbF. These patients are predominantly African American or of Asian ancestry, notably the same ethnicities that have increased prevalence of diabetes [15,16]. By some estimates, there are >335,000 diabetic patients in the US with hemoglobin variants [17]. The NGSP maintains a website www.ngsp.org [18] with information about specific instrumentation and reports of interference by non-A hemoglobins. Of the 36 methods detailed at the website, none have been proven to have zero interference from non-A hemoglobin. Individuals homozygous for HbS, C, and E, and compound heterozygotes of these should not be monitored by HbA_{1c}, as these conditions alter the life span of the red blood cell, leading to false interpretations of the result [8]. Individuals that are homozygous for HbS, C and E and compound heterozygotes have no HbA, and therefore IE-HPLC cannot quantitate HbA_{1c}. Although immunoassay and boronate affinity HbA_{1c} methods may produce results for these patients, these results do not accurately reflect glycemic control due to decreased red cell survival.

The major Hb variants can be identified by hemoglobin electrophoresis, isoelectric focusing, and HPLC, however the standards of care for diabetes do not recommend testing for variant hemoglobins. This is problematic, because heterozygotes with HbAS, HbAC and HbAE are asymptomatic. The National Institutes of Health recommends checking a patient for hemoglobinopathy when HbA_{1c} results are unexpected based on patient data, when a change in methodology produces a large change in HbA_{1c} result, or when the HbA_{1c} result exceeds 15% [19]. These guidelines imply that the clinician is cognizant of the methodology used, is adept at detecting discrepant results, and that all spurious HbA_{1c} results are grossly elevated. The National Academy of Clinical Biochemistry (NACB) guidelines for reporting HbA_{1c} recommend that laboratories should consider their particular patient population when selecting a method, and verify specimens with HbA_{1c} results below the lower limit of the reference interval or >15%. Furthermore, the NACB recommends that the laboratory makes physicians aware if the assay method in use is affected by hemoglobinopathies [20].

Many Hb variants can be detected during HbA_{1c} testing by IE-HPLC. Depending on the retention time and concentration, the variants may also be identified on the Tosoh G7 [17], Bio-Rad Variant II [21] Bio-Rad Variant II Turbo, Bio-Rad D-10, and other analyzers. Based on the peak information and manufacturer instructions, HbA_{1c} results can be accepted or rejected in the presence of the variant [10].

Any particular individual may have testing done by multiple methods as a hospital patient, an outpatient, at point-of-care, when insurance changes necessitate change of laboratory services or doctors, or when a laboratory changes its method. Selection of the best assay for a particular patient and consistency in testing is a feasible goal. A standardized report for HbA_{1c} that includes methodology and the presence of variant hemoglobins would improve the value of the HbA_{1c} report for patients with non-A hemoglobins. This project investigated common reporting practices in US laboratories with respect to reporting methodology, reporting variant hemoglobins, and recency of changes of methodology. Examples of best practices are included in this report.

2. Materials and methods

An article which included a survey on reporting practices for HbA_{1c} was published in LabMedicine [22] in July 2008. The survey was also e-mailed to all of the US directors of NAACLS approved MT/CLS and MLT/CLT programs. Survey questions included institution name, city, state, zip code, HbA_{1c} methodology (instrument and manufacturer). Reporting practices were investigated with this question: "When you report HbA_{1c} values do you (select one): A. state only the HbA_{1c} as a percentage, B. include the value and the methodology, C. include a statement if a hemoglobin variant has been detected, or D. other format (include details)." Recent method changes were investigated with this question: "Has your laboratory changed HbA_{1c} methodology in the last 2 years?" Comments and examples were encouraged. See Supplementary material for full survey.

One hundred forty-seven responses were received from hospitals, reference laboratories, and physician offices in the US, Canada, Uganda and the Bahamas. Only responses from US clinical laboratories are analyzed in this report. One hundred thirty-five clinical laboratories in 42 states responded, with no response from Arizona, Colorado, Idaho, Montana, Nevada, Rhode Island, South Dakota and Wyoming. Separately, the methodology used by several US reference laboratories for HbA_{1c} testing was determined by viewing each laboratory web page.

3. Results

Of 135 survey respondents, 61.5% report only the HbA_{1c} value and reference interval, with no information about methodology or Hb variants. The number of laboratories that routinely report test methodology was 7/135 (5%). One laboratory states the method only if a secondary method is required to determine accurate results. 99 of the laboratories (73%) responded that they had not changed methodologies in the past 2 years.

Sixty-eight respondents utilize IE-HPLC (51%), 64 utilize Immunoassay (47%), 2 use boronate affinity chromatography (BA) (1%), and 1 uses a dual system of BA and HPLC. Twenty seven of the 69 laboratories that use IE-HPLC (39%) routinely report the presence of hemoglobin variants in patient results. Seven laboratories using IE-HPLC (10%) mention the presence of a variant only if the variant interferes with the test. Variants are identified, e.g. "presence of HbS detected" only if the method has been validated to provide identities. Immunoassays and boronate affinity chromatography cannot discriminate variant hemoglobins, and have the common drawback that glycated HbF is not accounted for. Only 1 laboratory out of 64 that uses immunoassay relays this information, with the appended statement "Individuals with elevated HbF, chronic or significant recent blood loss must be assayed by an alternate method." Only 2 laboratories in the survey use boronate affinity chromatography, and neither laboratory appends a statement of limitations. Table 1 lists seven reference laboratories, including one laboratory that exclusively tests HbA_{1c}. The table lists the information available on their web pages with respect to methodologies and limitations.

Follow up protocols and comments were not analyzed here, since these were invited but not required as survey questions. Anecdotally, three laboratories reported crediting the HbA_{1c} when the specimen required follow up testing by a reference laboratory. Nine laboratories, including the reference laboratories, report HbA_{1c} methodology in their electronic laboratory manual. Fourteen laboratories reported that they suggest follow up for patients with variant hemoglobins, including sickle screens, hemoglobin electrophoresis, recommendation of the use of fructosamine as an alternate means of monitoring glycemic control, or testing for Glycohemoglobin by boronate affinity chromatography. Six laboratories, including the reference laboratories, reported that they include the test disclaimer that conditions that lengthen or shorten erythrocyte survival will cause erroneous results. No laboratory reported offering a reflex test for hemoglobin phenotype. The ethnic mix of the patient population affects how relevant laboratories view this issue. Two survey respondents commented that the populations they serve are predominantly white, and hemoglobinopathies are a rare finding, and not at the top of the list of important issues. Conversely, a laboratory from a major city reported that 95% of its population is African American, and that 1–2 hemoglobinopathies are seen daily; interference by variant Hemoglobin is viewed as a serious issue for that laboratory.

Three reporting practices stood out as best practices. One laboratory in the Southeast utilizes a dual assay approach, IE-HPLC to identify variants, and boronate affinity chromatography to determine glycohemoglobin. The patient result includes information about the methodology, the presence or absence of hemoglobin variants, and an estimated average glucose. Results from a patient with Hemoglobin C trait are shown in Fig. 1.

Table 1
Details available for reference laboratories reporting HbA_{1c}.

Name	Method listed	Limitations listed on web page, verbatim
Lab Corporation of America	Roche Tina Quant (Immunoassay)	"Any cause of shortened erythrocyte survival will reduce exposure of erythrocytes to glucose with a consequent decrease in Hb A_{1c} (%). Causes of shortened erythrocyte lifetime might be hemolytic anemia or other hemolytic diseases, homozygous sickle cell trait, pregnancy, or recent significant or chronic blood loss. Glycated Hb F (fetal hemoglobin) is not detected as it does not contain the glycated β chain that characterizes Hb A_{1c}. Specimens containing high amounts of Hb F (>10%) may result in lower than expected Hb A_{1c}."
Mayo Medical Laboratories	Bio-Rad Variant II Turbo IE-HPLC	"Most common hemoglobin (Hb) variants (HbF < 15%, HbC, HbS) do not interfere with this method. Other variants of Hb may show interference with this method. The known variants that fall into this category are hemoglobin E, Fukuoka, Philadelphia, Punjab, and Raleigh. In patients with rare homozygous forms (CC or SS) there is no hemoglobin A present and thus no Hb A_{1c} value can be quantitated using this method. If the specimen cannot be analyzed using this method due to a hemoglobinopathy, a second-tier test will be performed on the Bio-Rad in2it analyzer — a point-of-care National Glycohemoglobin Standardization Program (NGSP)-certified method utilizing boronate affinity chromatography, which is least affected by hemoglobin variants." "Red cell survival directly influences Hb A_{1c} concentration; falsely high values can be obtained when red cell turnover is low (e.g., post-splenectomy) and falsely low values when red cell turnover is high (e.g., hemolysis). Falsely high values may also be obtained when red cell mass in increased (e.g., polycythemia) and in iron deficiency anemia (mechanism not well-described). Treatment of these disorders may result in changing Hb A_{1c} concentrations that are unrelated to glucose control. Caution should be exercised when interpreting the Hb A_{1c} results from patients with these conditions."
Esoterix	Affinity chromatography HPLC	None listed
ARUP Laboratories	High performance liquid chromatography/boronate affinity	"This boronate affinity Hb A_{1c} method provides accurate analytical results in the presence of nearly all hemoglobin variants. Conditions that shorten red cell survival, such as the presence of unstable hemoglobins like Hb SS, Hb CC, and Hb SC, or other causes of hemolytic anemia may yield falsely low results. Conditions like iron deficiency anemia may increase red cell survival and yield falsely high results."
Quest Diagnostics	Immuno-turbidimetry	None listed on ordering page. Interpretive guide states "Increased: diabetes mellitus, chronic hyperglycemia, presence of hemoglobin S, presence of hemoglobin C variant. Decreased: relatively decreased by improved diabetic control, high levels of hemoglobin F."
DTI Laboratories, Inc.	Multi-method (HPLC-IE and HPLC-BA).	"The two step process includes a screening step to detect hemoglobin variants and/or disturbed erythrocyte kinetics by HPLC-ion exchange (IE). The second step includes the use of an interference-free procedure HPLC-boronate affinity (BA), that provides an A_{1c} value free of possible interferences including chemically modified derivatives."
Specialty Laboratories	Primus boronate affinity	"The boronate affinity method for the determination of glycated hemoglobins is not affected by the presence of abnormal hemoglobin variants".

A laboratory in New England described the algorithm used to evaluate IE-HPLC results for HbF, HbS, HbC and other peaks for suitability to release results. The algorithm is shown in Fig. 2. The presence of variant hemoglobins is communicated to the physician by standard statements on the report, and the laboratory director communicates with the ordering physician in the case of results that cannot be discerned.

Another laboratory in the Pacific Northwest tests all new patients using IE-HPLC. If no variants are detected, point-of-care immunoassay testing is permissible. Point-of-care testing is under laboratory control in this health care system.

4. Discussion

Efforts to standardize HbA_{1c} testing are international. A consensus statement endorsed by the International Federation of Clinical Chemistry, the ADA, the European Association for the Study of Diabetes and the International Diabetes Federation states that HbA_{1c} results should be reported both in the well-established NGSP units (%) and newer IFCC units (mmol/mol Hb) [23,24]. The American Association for Clinical Chemistry (AACC) has recently endorsed the inclusion of an estimated average glucose as part of the HbA_{1c} result [25]. There is, however, no IFCC recommendation for a standardized HbA_{1c} report that includes methodology or its possible effect on results from a patient with hemoglobinopathy, in spite of the 2002 NACB recommendation that physicians be informed of HbA_{1c} methods with hemoglobinopathy interference. The survey reported here found no standard format that informs a clinician that his/her patient has a hemoglobin variant or what method the laboratory has used. Most laboratories responding to the survey do not give any information about method or Hb variants. This is an opportunity for improving standardization.

Each diabetic patient with a hemoglobinopathy has a potential for an inaccurate HbA_{1c} result depending on the method used. The inaccurate result impacts the patient, the physician and the laboratory. Patient treatment is adjusted based on HbA_{1c} and glucose values. Patients with higher than recommended average glucose risk micro and macrovascular complications. Patients with lower than recommend average glucose risk hypoglycemia and its associated risks.

The physician's order for HbA_{1c} testing does not specify a method. It is reasonable for the clinician to believe that all HbA_{1c} tests are created equal. Physicians are not good at assessing glycemic control based on patient history [26], yet according to the NIH guideline they are expected to recognize unexpected results. Laboratory selection is frequently dictated by insurance contracts. One physician may deal with several testing laboratories, as well as his or her own point-of-care method. The physician is expected to be aware of changes in HbA_{1c} methodology for each laboratory and patient, and to be tuned into test limitations that may or may not be listed in laboratory electronic or written manuals, and rarely stated on test reports.

Patients with variant hemoglobins impact the laboratory in quality of result, time and cost. When a method cannot detect an interfering hemoglobin, the result is a medical error. Laboratories capable of detecting Hb variants and/or Hb F have established their own mechanisms to deal with these samples. These include repeating the test, testing the sample with a sickle screen, running hemoglobin electrophoresis, sending the sample out to a reference laboratory or running it on an alternate methodology in-house, phoning the physician, and filing information in a database. Without a mechanism to identify this patient as having a Hb variant, this cycle is repeated when the patient returns 3 months later. It is not unusual for a patient to move to another geographic location, or change laboratories due to changes in health coverage. This may result in a chain of events to re-determine the patient's Hb phenotype. With each transfer, the responsibility falls on the laboratory to produce the accurate results. Repeat testing of HbA_{1c} in patients with interfering hemoglobin variants results in extra costs for the laboratory that in turn may lead to extra cost to the patient.

Several of the reference laboratories shown in Table 1 analyze HbA_{1c} by immunoassay or boronate affinity chromatography. While specific methods have proven accurate in patients that are heterozygous for the

Test Description	Results	Units	Range	
Hemoglobin A1c (HbA1c):	8.0	%	4.2 - 6.0	H
estimated Average Glucose (eAG):	183	mg/dl	73 - 125	H
Abnormal peak and/or silent variant:	YES	See comments		

estimated Average Glucose is derived using the equation: (28.7 X HbA1c – 46.7)= eAG mg/dl

Comments:
One or more abnormal peaks consistent with a hemoglobinopathy were detected, further diagnostic testing is recommended. HPLC-high resolution chromatography demonstrated a peak consistent with hemoglobin C (HbC). This individual/physician should be advised that various A1c testing method may provide an interference to this variant.

HPLC-boronate affinity (BA) was used to determine an interference-free A1c calculation.

Each 1 % increase in HbA_{1c} is associated with an increase ineAG of approximately 29 mg/dl.

A_{1c} test results should be interpreted and target levels set by a healthcare professional. The American Diabetes Association (ADA) recommends maintaining A_{1c} levels below 7.0%.

Method of Analysis:
This sample was analyzed using 2 separate methods of analysis:
High Performance Liquid Chromatography (HPLC-IE) or Low/High Resolution Chromatography for the Detection of silent (abnormal) variants and/or abnormal peaks, and;
High performance Liquid Chromatography (HPLC) boronate affinity (BA) to determine an interference-free A1c value.

Fig. 1. Laboratory report. Test results include presence or absence of variant. Comments section describes Hb variant. Method of analysis described sample testing.

major hemoglobinopathies (S, C, E, D), immunoassays and boronate affinity chromatography can produce spurious results in the presence of high HbF [11]. Neither immunoassay nor boronate affinity chromatography can detect the presence of HbF or quantitate it. IE-HPLC can detect the presence of HbF, and based on manufacturer determined cutoff values, HbA_{1c} results can be reported [10]. There is a lack of scientific review of HbF; of the 36 HbA_{1c} methods listed at the National Glycohemoglobin Standardization Program website, 26 methods had

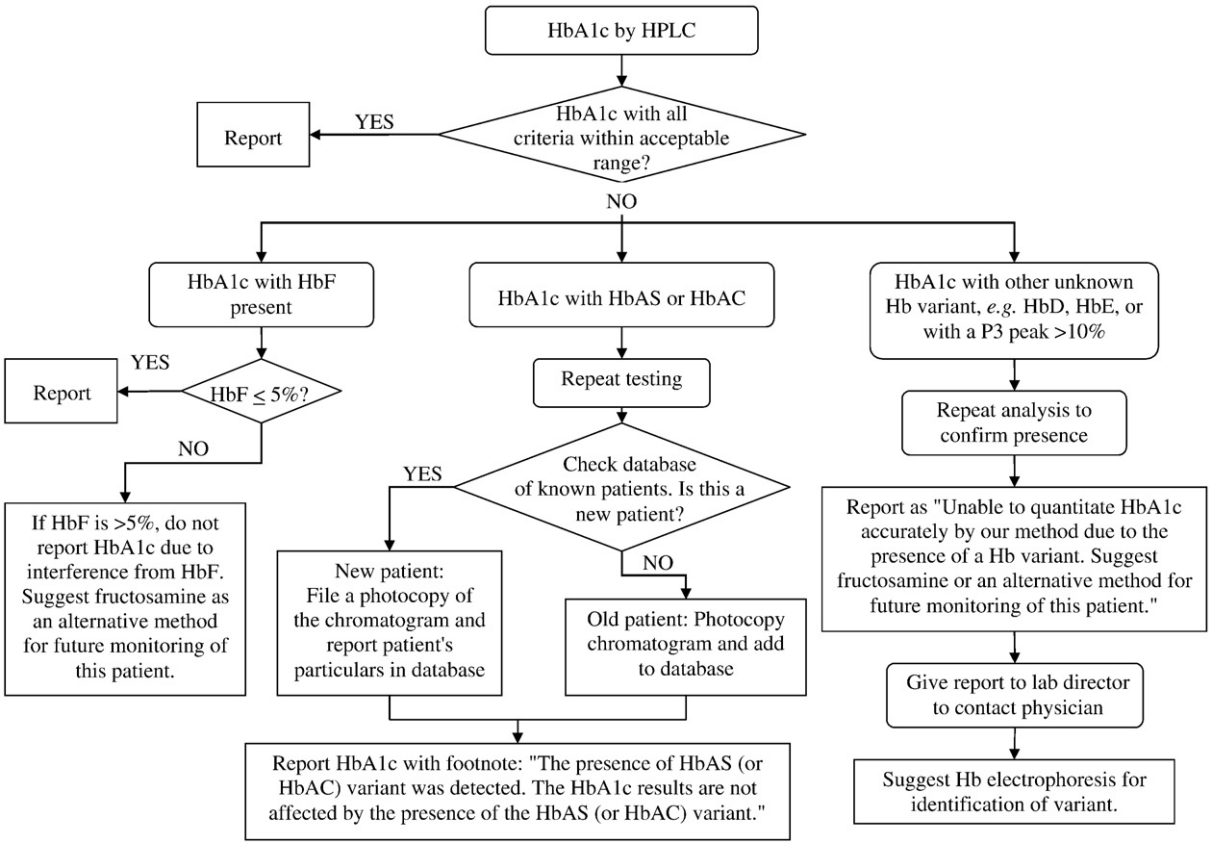

Fig. 2. Accept/reject criteria for HbA_{1c} in the presence of a variant Hb.

not been evaluated to determine if HbF interferes with the results. HbF usually constitutes <2% of the total hemoglobin. Elevated amounts of HbF, in amounts high enough to interfere with HbA_{1c} assays are found in patients with homozygous β^+ or double heterozygous β^+/β^0 thalassemia, heterozygous $\delta\beta$ thalassemia, and in patients with heterozygous Hereditary Persistence of Hemoglobin F (HPFH), both the African and Greek type. In all of those conditions, HbA is also present. HbF is also elevated, but HbA is absent in homozygous β^0 thalassemia, homozygous $\delta\beta$ thalassemia, and homozygous HPFH [27]. HbF may also be elevated in heterozygotes of β thalassemia and other hemoglobinopathies, e.g. HbS/β^+ thalassemia [22,27], and approximately half of the carriers of β thalassemia have mildly elevated HbF [27]. HbF was the most commonly identified non-A hemoglobin in Project DIRECT, a study done with predominantly African American subjects [28]. In that study, over 25% of the African American participants had Hb variants. Early identification of a patient with increased HbF can divert that patient to a method that is more appropriate. Although HbF is quantitated by IE-HPLC, only 3 survey respondents that utilize IE-HPLC included HbF percent as part of the routine HbA_{1c} result.

Physiologic conditions that alter RBC survival, including iron deficiency anemia, sickle cell disease, and hemolytic diseases can alter the relationship between average glucose and HbA_{1c}, causing a false low or high HbA_{1c} [8,29,30]. These conditions were not explored in this study. Higgins recommended using complete blood count (CBC) parameters and recent glucose results to evaluate unexpectedly low or high HbA_{1c} results [31].

The presence of non-A Hemoglobins can alter the relationship between average glucose and HbA_{1c} subtly, producing results that are within the expected interval. A standardized reporting format based on the best practices compiled here can reduce erroneous HbA_{1c} results due to the common Hb variants. The HbA_{1c} report should include the methodology used, the presence/absence of Hb variants, and the % of HbF when available. A statement of the limitations of the method should be included on the report, and be published in the electronic laboratory manual.

Interference due to variant hemoglobins and HbF is an international problem. It is estimated that 11% of African Americans have Hb S or Hb C. In some parts of Africa the prevalence is as high as 20–40% [32]. Hb E is the third most common Hb variant in the world, associated with Southeast Asia [32]. Hemoglobinopathies and thalassemias are common in the Mediterranean countries, in India, in Africa and in Southeast Asia, and are found in immigrants and their descendents from these areas worldwide. Manley et al. [10] proposed a strategy to determine which HbA_{1c} methods would be appropriate for patients based on their likelihood of having a variant, based on ethnicity, geographical location or clinical situation. A best practice reported here is to determine Hb phenotype on all patients early in their diabetes management as part of the standard of care. The Hb phenotype becomes part of the patient electronic medical record, and is integral in selecting which HbA_{1c} method is utilized for that patient.

Implementing a change in result reporting, i.e. reporting the limitations of the test, and the presence of Hb variants when available necessitates increased communication between the laboratory and the medical staff, and may bring about an increase in orders for Hb phenotype. The inclusion of a statement that addresses the impact of Hb variants on results will prevent inaccurate result interpretation, as well as the cycle of discovery/retest/cancel/credit that occurs when the same patient returns for testing. This will save costs to the testing laboratory and patient, it will have the benefit of decreasing the turn-around-time from sample collection to result, and assure its accuracy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cca.2009.06.012.

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