Use of Fetal Hemoglobin Quantitation for Rh-Positive Pregnant Females

A National Survey and Review of the Literature

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• ***Context.***—**The Kleihauer-Betke (KB) test is validated for estimating the dose of Rh immune globulin needed for Rh-negative pregnant females. However, some clinicians are also ordering the test for Rh-positive women. The degree to which this practice occurs is unknown.**

Objective.—**To evaluate the number of laboratories that perform the KB test on Rh-positive pregnant women, and to establish current ordering practices for this indication.**

Design.—**We added 9 supplemental questions regarding KB test use for fetomaternal hemorrhage to the 2016 College of American Pathologists proficiency test survey. We also reviewed the available literature regarding the diagnostic utility of the KB test for Rh-positive women.**

Results.—**A total of 1578 surveys were evaluated and revealed that 52% (824) of respondents perform these tests for Rh-positive women, and more than 50% (440 of 819; 53.7%) of these laboratories report that the results for Rh-positive women are treated as important or very important.**

Conclusions.—**The KB test is commonly used for Rh-positive women, and the information obtained from the test is considered as urgent and important. However, the available literature in support of this practice is still nonconclusive.**

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In the United States, nearly all (96.0%) quantitative assays for fetal red blood cells in maternal circulation are performed using several commercially marketed kits for an acid-elution assay, also known as the Kleihauer-Betke (KB) test.[1] Although it has been validated to estimate the amount of Rh immune globulin (RhIg) to provide an Rh-negative pregnant female,[2] a controversial report suggests that this test may also be helpful to identify and quantitate fetal red blood cells in circulation for RhD-positive (Rh+) females in cases of suspected fetomaternal hemorrhage.[3] Particularly, some guidelines suggest that a KB test should be performed urgently on all pregnant females who present with trauma in order to predict actionable morbidity.[3] The degree to which these guidelines have been incorporated into routine clinical practice is unknown. Consequently, the focus of our survey was to establish the prevalence of laboratories that perform KB tests on Rh+ females, and the degree to which these laboratories understand their diagnostic role for these patients. Further, we reviewed the available literature regarding the clinical utility of a positive KB test for this patient population.

METHOD

The College of American Pathologists proficiency testing program offers samples for fetal red blood cell detection to laboratories that perform this test. Twice each year, 2 specimens are provided with each proficiency test. Participants can screen the specimens with a fetal rosette test and also determine the percentage of fetal red blood cells present by either a staining (KB test) or a flow cytometric technique.

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sought to determine to what extent participating laboratories performed these tests specifically for Rh+ pregnant females. To this end, the College of American Pathologists committee added 9 supplemental questions to the 2016 Hemoglobin F, mailing B, proficiency test.[1] These added questions were as follows: (1) Does your laboratory perform fetal bleed quantitation testing in Rh+ women with risk for fetomaternal hemorrhage? (2) In what time frame does your laboratory provide the test results? (3) How many deliveries does your center do per year? (4) How many fetal bleed quantification tests does your laboratory do per year for the alternative (Rh+) indication? (5) How many fetal bleed quantification tests does your laboratory do per year for RhIg dosing (standard indication)? (6) What is the turnaround time published by your laboratory to complete an urgent (or "STAT") fetal bleed quantification test after specimen arrival in the laboratory? (7) What department orders the fetal bleed quantification test for the alternative indication the most? (8) What type of providers order the fetal bleed quantitation test for the alternative indication? (9) When the fetal bleed quantitation is ordered for the alternative indication, how do the results of the testing guide the management of the mother and fetus? These additional survey questions could be answered by any appropriately trained laboratory staff member, per the proficiency test instructions. Respondents were allowed to involve their medical director, as needed, to assist with the more clinical questions.

There were 1696 survey responses received, and 118 were excluded prior to analyzing the data for the following reasons: duplicate survey submissions (n = 6), partial survey submissions (n = 20), and reference laboratory designation (n = 92). Reference laboratories were specifically excluded from the analysis because of a lack of applicability for the majority of survey questions. Responses that did not follow the survey's skip sequence directions were also adjusted for data validity. A total of 1578 surveys were included in the summary, for a 76% response rate from the 2067 laboratories surveyed.

Statistical analyses using Pearson χ^2 tests were performed to test for practice characteristics significantly associated with KB testing in Rh+ women. These characteristics included laboratory location, annual number of deliveries, and annual volume of KB testing for the standard indication. Only one practice characteristic was significantly associated with KB testing practice, so no multivariate models were evaluated. A P value $<.05$ was considered statistically significant. The survey results summary and analyses were generated with SAS 9.4 (SAS Institute, Cary, North Carolina).

RESULTS

A total of 1578 laboratory surveys were analyzed, and the majority of respondents (93.5%; 1476) were from the United States. Most of the 1149 laboratories that reported a fetal red blood cell count result performed fetal hemoglobin quantitation using a modified KB test (acid-elution test; 1099; 95.6%), and 50 laboratories (4.4%) reported using flow cytometry. More than half of the evaluated participants (824; 52.2%) noted that they performed fetal hemoglobin quantitation for Rh+ women, and about 39% (308 of 789) of those reporting sites performed more than 20 tests a year (Table 1). The evaluated laboratories represented a diverse group of clinical practices; hospitals with more than 500 and

Table 1. Selected Survey Responses From the Fetal Red Blood Cell Detection College of American Pathologists Proficiency Test Supplemental Questions

Survey Question	No. (%)
Laboratory performs fetal bleed quantification testing in RhD-positive women with risk for fetomaternal hemorrhage (ie, patients experiencing trauma or placental abruption)	n = 1578
Yes	824 (52.2)
No	754 (47.8)
No. of fetal bleed quantification tests done per year for the RhIg dosing (standard indication)	n = 1259
≤20	850 (67.5)
21–50	178 (14.1)
51–100	119 (9.5)
101–200	67 (5.3)
>200	45 (3.6)
No. of fetal bleed quantification tests done per year for the alternative indication	n = 789
≤20	481 (61.0)
21–50	139 (17.6)
51–100	100 (12.7)
101–200	45 (5.7)
>200	24 (3.0)
Time frame in which laboratory provides the test results for the alternative indication, h	n = 823
<6	623 (75.7)
6–12	92 (11.2)
12–24	97 (11.8)
>24	11 (1.3)
Department that orders the most fetal bleed quantification tests for the alternative indication	n = 816
Obstetrical or birthing department	461 (56.5)
Emergency department	280 (34.3)
Other	14 (1.7)
Not sure	61 (7.5)
Type of providers who order the fetal bleed quantification test for the alternative indication	n = 815
Obstetricians, midwives	445 (54.6)
Emergency room providers	266 (32.6)
Primary care providers, family medicine, and/or internal medicine	12 (1.5)
Other	9 (1.1)
Not sure	83 (10.2)
When the fetal bleed quantification is ordered for the alternative indication, how do the results of the testing guide the management of the mother and fetus?	n = 819
Always treated as very important information	301 (36.8)
Often treated as very important information	139 (17.0)
Usually not treated as very important information	15 (1.8)
Other	2 (0.2)
Unsure of how results are used by the ordering provider	362 (44.2)

Abbreviation: RhIg, Rh immune globulin.

Table 2. Association Between Kleihauer-Betke Testing for RhD-Positive Women and Key Laboratory Characteristics

	Alternative Indication Performed, No. (%)	Alternative Indication Not Performed, No. (%)	*P* Value[a]
Laboratory location[b]			
United States	765 (92.8)	711 (94.3)	.24
Northeast	150 (19.7)	108 (15.2)	
Midwest	172 (22.5)	164 (23.1)	
South	269 (35.3)	275 (38.7)	
West	172 (22.5)	163 (23.0)	
Outside the United States	59 (7.2)	43 (5.7)	
Annual No. of deliveries			
≤500	156 (19.9)	258 (37.3)	<.001
501–1000	147 (18.8)	178 (25.8)	
1001–5000	427 (54.5)	236 (34.2)	
5001–10 000	54 (6.9)	19 (2.7)	
Annual volume of KB testing for the standard indication			
≤20	525 (65.0)	325 (72.1)	.06
21–50	122 (15.1)	56 (12.4)	
51–100	78 (9.7)	41 (9.1)	
101–200	48 (5.9)	19 (4.2)	
>200	35 (4.3)	10 (2.2)	

Abbreviation: KB, Kleihauer-Betke.

[a] Chi-square test.
[b] Regions are based on US Census Bureau definitions.[44]

up to 10 000 deliveries were represented, and institutions with higher-volume delivery centers performed KB testing for Rh+ females significantly more frequently than lower-volume centers (Table 2). Requests for this testing primarily came from obstetricians or midwives (445 of 815; 54.6%), followed by emergency department providers (266 of 815; 32.6%). Accordingly, the majority of 816 participants noted that requests for alternative fetal hemoglobin quantitation testing came from obstetrical/birthing departments (461; 56.5%), followed by emergency departments (280; 34.3%). More than half of the 819 participants (440; 53.8%) noted the results of this testing were reported as always or often being treated as very important information for patient care, and the majority of 823 participants noted that turnaround time for this testing was expected to be completed in less than 6 hours (623; 75.7%). Importantly, 44.2% (362 of 819) of participants stated they were unsure how the results of the test were used by the ordering provider.

DISCUSSION

Supplemental questions appended to College of American Pathologists proficiency test surveys have been a valuable source of information on national laboratory and medical practices.[4–8] The current survey demonstrates that KB testing for Rh+ women is commonly ordered, especially in larger hospitals, and is generally treated as important clinical information, but the reason for the order is unclear for a significant proportion of laboratories.

The noted confusion among laboratory professionals is understandable, as the use of KB testing for Rh+ pregnant women has been rarely a focus of study, and much of what we know regarding the sensitivity and specificity of KB methods is for Rh-negative women.[2] Although it has been validated for RhIg dosing, proficiency surveys and other evaluations of the acid-elution assay consistently find it lacking precision, with a wide range of interoperator and intraoperator variation.[2] Accuracy has been shown to be very operator dependent and has a reported error rate of up to 50% when positive.[9]

A number of technical and patient factors impact the accuracy and reproducibility of the KB test.[10,11] First, the KB test can underestimate the amount of fetomaternal hemorrhage if fetal cells fail to stain.[12,13] The KB protocol itself is notoriously complicated and time intensive,[13] and relatively minor variations in test performance can lead to unexpectedly large variations in results. Anecdotally, blood smears that are too thick, slides that are not allowed to adequately dry, and inappropriately short buffering times can all lead to fetal cell understaining. Second, underestimation may also occur because of the known decreasing hemoglobin F (HbF) content of fetal erythrocytes with advancing gestational age.[13] By term, HbF may represent only 70% of the hemoglobin of the fetal red blood cell, and may be missed because of light staining.[13,14] Lastly, false-negative results could occur in situations where preformed antibodies, such as in ABO-incompatible pregnancies, result in the removal of fetal red cells by the maternal reticuloendothelial system before detection by a KB test.[13]

In contrast, overestimation may occur because maternal HbF can mimic the appearance of true fetal red cells and can lead to false-positive results. Among the medical conditions associated with an increased number of HbF-containing cells in adults are hereditary persistence of HbF, sickle cell anemia (with or without treatment with hydroxyurea), β-thalassemia, aplastic anemia, and certain other hematopoietic diseases associated with erythropoietic stress.[15–18] Maternal HbF-containing red cells are also known to increase naturally during pregnancy, compounding the problem.[16–18] In approximately 25% of pregnant women, HbF starts to increase after 8 weeks' gestation and may reach 7% by 32 weeks.[18,19] Lastly, 40% to 50% of all pregnancies late in gestation have some fetal red cells in circulation.[20] It is known that some fetal red cells normally enter the maternal circulation (usually less than 15 mL in most cases) during all pregnancies and circulate over a number of weeks without apparent clinical significance.[21] As a consequence, neither positive nor negative KB test results may be accurate, and positive KB tests, especially late in pregnancy, may not be a result of acute fetal injury or harm, even though injury may have been reported clinically.

As noted by our survey, the KB test is often urgently recommended for Rh+ pregnant women in trauma based on the assumption that the injury might be great enough to cause a transfer of fetal erythrocytes that would lead to actionable fetal morbidity from abruption or uterine injury. Previous studies have supported the notion that significant fetomaternal bleeding events are predictive of fetal mortality.[13] However, the evidence in support of the KB test result's being predictive of adverse fetal outcomes without other signs of fetal distress is lacking. We identified 16 observational studies evaluating 19 to 394 pregnant females that used the KB test as a predictive biomarker for fetal morbidity and mortality (Table 3). The majority of these studies likely used a combination of Rh+ and Rh-negative females, as only 3 (19%) of these studies actually defined the number of Rh+ females evaluated.[22–24] These studies also predominantly used fetal cell positivity (ie, any fetal cells

Table 3. Studies Evaluating the Predictive Value of the Kleihauer-Betke (KB) Test for Adverse Fetal Outcomes[a]

Source, y	Study Type	Pregnant Patient Population	Outcome(s) of Interest	Total No. of Subjects Evaluated With a KB Test	Total Rh-Positive, No. (%)	Total Positive KB Tests, No. (%)	Total Positive KB Tests With Adverse Outcome, No. (%)	Conclusion
Stroustrup and Plafkin,[22] 2016	Prospective cohort study	Healthy term pregnant women	Neonatal anemia	19	19 (100)	2 (10)	2 (100)	2 of 5 (40%) with neonatal anemia had a positive KB test
Atkinson et al,[33] 2015	Retrospective cohort study	Placental abruption	Placental abruption	68	NA	3 (4.4)	3 (100)	Not diagnostic
Trivedi et al,[25] 2012	Retrospective cohort study	All pregnant patients	Abnormal ultrasound Abnormal uterine activity Preterm labor/delivery Abruption Fetal death	167	NA	42 (25.1)	NA	Predictive of morbidity when combined with: Third-trimester trauma Abdomen trauma LOS >2 d
Huissoud et al,[23] 2009	Case-control	Women with large FMH (>20 mL)	Fetal death Postnatal death Severe neonatal anemia (<6 g/dL)	32	29 (91)	Cases: 6.5% fetal cells Controls: 0.5% fetal cells	NA	Diagnostic when >2.5%
Cahill et al,[26] 2008	Prospective cohort study	Minor trauma	Abruption Preterm delivery Birth weight <10th percentile	317	NA	9 (2.8)	2 (22.2)	Not diagnostic
Dhanraj and Lambers,[34] 2004	Case-control	Varying degrees of trauma	Fetal distress Placental abruption	249	NA	9 (2.6% of trauma patients)	0 (0)	Not diagnostic
Muench et al,[9] 2004	Retrospective cohort study	Varying degrees of trauma	Preterm labor	71	NA	46 (65)	25 (54.3)	Diagnostic for preterm labor and uterine contractions
Pak et al,[27] 1998	Prospective cohort study	Blunt abdominal trauma	Preterm delivery	85	NA	0 (0)	0 (0)	Not diagnostic
Connolly et al,[28] 1997	Retrospective cohort study	Varying degrees of trauma	Preterm labor Preterm delivery Abruption Birth weight <2500 g	289	NA	63 (21.8)	10 (15.9)	Not diagnostic
Emery et al,[29] 1995	Retrospective cohort study	Patients with a KB test	Placental abruption Preterm labor	394	NA	22 (5.6)	1 (4.5)	Not diagnostic
Dahmus and Sibai,[30] 1993	Retrospective cohort study	Noncatastrophic blunt abdominal trauma	Abruption placentae Preterm labor Fetal distress Fetal death	99	NA	21 (21.2)	1 (4.8)	Not diagnostic
Towery et al,[31] 1993	Retrospective cohort study	Varying degrees of blunt trauma	Uterine contractions Abortion Placental abruption Preterm labor Fetal demise Vaginal bleeding Fetal heart deceleration	87	NA	30 (34)	8 (27)	Not diagnostic

Table 3. Continued

Source, y	Study Type	Pregnant Patient Population	Outcome(s) of Interest	Total No. of Subjects Evaluated With a KB Test	Total Rh-Positive, No. (%)	Total Positive KB Tests, No. (%)	Total Positive KB Tests With Adverse Outcome, No. (%)	Conclusion
Dupre et al,[36] 1993	Prospective cohort study	All women ≥20 weeks gestation with risk factors (eg, trauma or substance abuse)	Neonatal anemia	65	NA	14 (22)	2 (15)	Not diagnostic for fetal outcome
Pearlman et al,[32] 1990	Prospective case-control study	Varying degrees of trauma	Abruptio placentae Rupture of membranes Onset of labor Fetal death	170	NA	33 (30.6% of trauma patients)	1 (3.0)	Not diagnostic
Holcomb et al,[35] 1990	Retrospective cohort study	Patients who get a KB test; mixed indications	Neonatal anemia Fetal death	205	NA	18 (8.8)	2 (11)	Not diagnostic
Goodwin and Breen,[24] 1990	Prospective case-control	Noncatastrophic trauma	Premature labor Placental separation Fetal injury Fetal death	315	20 (8.8% of trauma patients)	NA	0 (0)	Not diagnostic

Abbreviations: FMH, fetomaternal hemorrhage; LOS, length of stay; NA, not applicable.

[a] All studies reported here used the manual or slide method, with the exception of Cahill et al,[26] where flow cytometry was reported toward the end of the study.

detected) as the predictor of interest (only 1 of 16 used the predicted percentage of fetal bleed).[23] Given these caveats, we found that only 3 (19%) of the 16 studies identified the KB test as predictive of adverse fetal outcomes.[9,23,25] Trivedi et al[25] defined any positive KB test (regardless of degree of positivity) to be predictive of adverse fetal outcomes when paired with the occurrence of maternal trauma to the abdomen and a hospital stay greater than 2 days. Huissoud et al,[23] in a case-control study, identified a 2.5% or greater fetal bleed as defined by the KB test to be predictive of fetal death, postnatal death, or neonatal anemia. Lastly, Muench et al[9] identified a positive KB test as predictive for preterm labor. Of the remaining studies, 8 found no association between the KB test and preterm delivery or labor,[24,26–32] 8 found no association with placental abruption,[26,28–34] 5 found no association with fetal mortality,[24,30–32,35] and 3 found no association with neonatal anemia.[22,35,36] Taken together, the current literature is at best not conclusive, and the significance of the KB test result and its utility as a predictor for fetal outcomes is questionable.

Because of the lack of primary evidence, expert guidelines also remain somewhat mixed regarding how the KB test should be used for Rh+ pregnant women in acute situations. In one guideline, Brown[37] documents that there is little evidence that KB testing predicts adverse outcomes, and that abnormal heart findings would be more predictive of fetal jeopardy. In contrast, Muench and Canterino[3] state that a KB test should be considered in all trauma patients, regardless of Rh status, because it may be an indicator of the severity of uterine trauma and an indicator of those patients at risk of preterm labor. Murphy and Quinlan[38] also assert that the KB test should be performed after major trauma to determine the degree of fetomaternal hemorrhage in all pregnant women, regardless of Rh status. Even major medical textbooks such as *Pediatric Emergency Medicine* by Baren et al[39] recommend the use of KB testing for all pregnant trauma patients, and cite the study of Muench et al[9] as evidence for this practice. Lastly, Wylie and D'Alton[13] assert that, despite known limitations, the KB test should be used any time fetomaternal hemorrhage is suspected antenatally. These authors, however, admit that the turnaround time of this test may not be helpful in emergencies, and peripheral blood flow cytometry testing might be more helpful given the possible need for rapid intervention.

Unlike the rapid prognostic indications noted above, the KB test remains a gold-standard tool for all pregnant females when used to evaluate the presence of fetomaternal hemorrhage as a contributor to unexplained fetal demise. Fetomaternal hemorrhage is a cause of fetal death in an estimated 1.6% to 11% of reported cases,[40,41] and the definitive diagnosis of a lethal fetomaternal hemorrhage requires confirmation of significant fetal blood volume loss.[41] Although the critical volume required for death remains unknown, some studies suggest that as little as 20 mL/kg blood volume transfused to the mother's circulation may be sufficient.[13] Compared with the KB test, however, other available clinical and laboratory findings are even less diagnostic for this indication: maternal risk factors do not appear to predict an increased likelihood of massive fetomaternal hemorrhage,[40] and postmortem autopsy findings, such as nucleated red cells or placental pallor, have been shown in a number of studies to be suggestive but not pathognomonic for fetomaternal hemorrhage.[42,43] Consequently, the KB test can be diagnostically helpful in Rh+

females with unexplained fetal death, and can be useful even when drawn days after the event.[41]

CONCLUSIONS

The dosing of RhIg in Rh-negative pregnant females is an, albeit flawed, established and accepted use of the KB test. However, our survey of 1578 laboratories revealed that this test is often being used for rapid diagnostic or prognostic purposes in Rh+ pregnant females. Although only a minority of studies demonstrated any predictive value of the KB test in these patients, expert guidelines still cite this test as potentially useful, and these guidelines are likely driving the observed clinical practice. Clearly, larger, well-designed, adequately powered, and prospective studies will be needed to clarify whether KB testing has any prognostic role. That said, the KB test currently cannot meet the needs of a trauma patient: it cannot predict fetomaternal bleeds with reproducibility because of known technical and biological complexities, and it is not a test that can be done rapidly. Consequently, outside of its potential use as a diagnostic tool post–fetal demise, the KB test's utility to guide urgent clinical decision-making outside of RhIg dosing (which has a 72-hour window after exposure) is limited.

Laboratorians should work to educate providers regarding the limitations of current KB testing methodologies, and should urge providers to limit clinical decision-making based solely on results of this test outside of RhIg dosing.

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