Comparative Study Analysing the Method Change Validation Protocol for the Kleihauer-Betke Test Assessing Fetomaternal Haemorrhage

Matt Foxwell James Cook University Hospital South Tees NHS Foundation Trust

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Aim:

Validate the Guest Medical Kleihauer kit as a replacement for the Clin-Tech Kleihauer kit used for estimating Fetomaternal Haemorrhage (FMH) volumes.

Introduction

- FMH can be a serious condition for both mother and fetus depending on the timing and size of the transplacental bleed, with the primary concern being the development of Haemolytic Disease of the Fetus and Newborn (HDFN) (Figure 1) (de Haas *et al.,*).

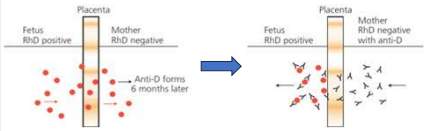

Figure 1 – HDFN pathogenesis due to RhD incompatability. RhD+ fetal RBCs cross the placenta in an FMH-sensitising event, causing maternal alloimmunisation. Upon subsequent RhD+ pregnancies, allogenic IgG anti-D antibodies cross the placenta and bind the D-antigen on fetal RBCs, marking them for destruction by extravascular haemolysis in the liver and spleen. Figure adapted from Contreras, 2009.

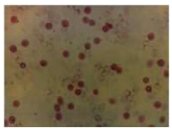

Figure 2 – Positive Kleihauer blood film. Fetal RBCs (red) can be seen populated among 'ghost' maternal RBCs (faded) from a peripheral maternal blood sample.

- HDFN prevention is a dose of prophylactic anti-D antibodies given to the mother, aiming to neutralise any circulating RhD positive fetal RBCs and therefore prevent alloimmunization, protecting later pregnancies (Qureshi *et al.,* 2014).
- Prophylactic anti-D dose is proportional to the estimated FMH volume. The size of a potential FMH is estimated by counting fetal RBCs in maternal circulation.
- The Kleihauer-Betke test is a differential blood staining procedure used to identify fetal red blood cells (RBCs) in maternal circulation (Figure 2) due to a fetomaternal haemorrhage (FMH) (Kleihauer, 1957).
- Mandated change from CE to UKCA marking and reduced cost per test necessitated the need for a Kleihauer kit change. Method change validation aims to determine agreement between the prospective instrument's performance compared to the established current methodology, and to the considered gold-standard method.

Methods

- Test samples were processed following Trust standard operating procedure (Figures 3 & 4).

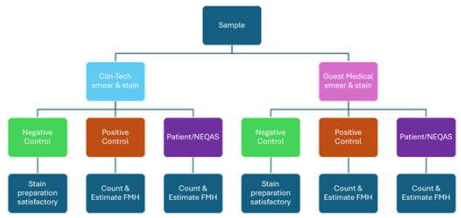

Figure 3 – Sample selection quantification flowchart. The samples were either aliquots taken from routine Kleihauer patients, spiked samples which were >2mL, or NEQAS samples.

- All slides were initially screened for presence of fetal RBCs. If fetal RBCs were present, the technician proceeded to a quantification (Kim and Maker, 2012).

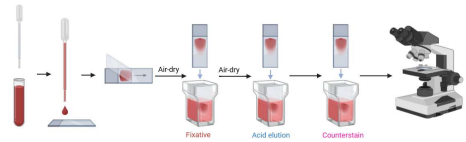

Figure 4 – Basic principle of the laboratory Kleihauer staining protocol. Each sample was prepared following the respective manufacturers guidelines. A drop of sample is added to a glass slide and smeared to create a monolayer. A dried slide is fixed in the manufacturer-provided fixative solution. The slide is fully air-dried before an acid elution step followed by rinsing, immediate counterstaining and microscopy for fetal RBC identification.

- Repeat FMH estimations were performed on all quantified samples.
- FMH volumes were calculated using the Mollison formula (Mollison, 1972).

Results

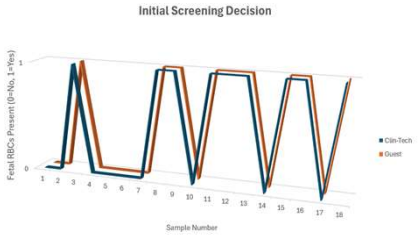

Figure 5 – Screening comparison. Slides were assessed through a binary yes/no method for decision to proceed to a quantification.

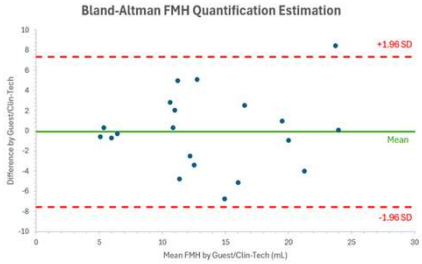

Figure 6 – Bland-Altman of spiked samples. Mean FMH volume estimated as measured by Guest and Clin-Tech versus the difference in FMH estimation between the two methods. Limits of agreement (LoA) were set at 95% confidence intervals +/- 1.96 from the mean of results.

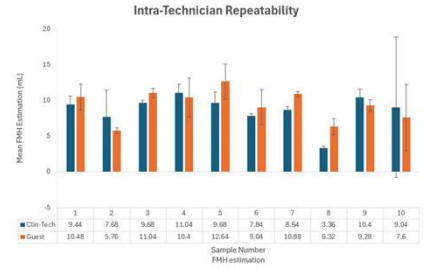

Figure 7 – Repeatability interpretation. Bar graph displaying the mean FMH estimation of the positive control samples by either method from 3 repeat counts by a single technician. The error bars represent the standard deviation.

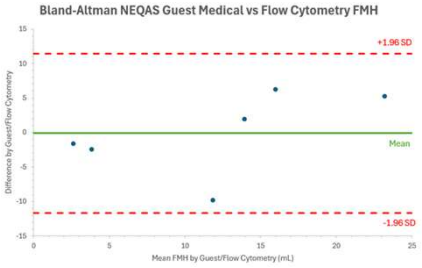

Figure 8 – Bland-Altman plot demonstrating agreement between Guest Medical manual Kleihauer and the gold standard flow cytometry (FC). Mean FMH volume estimated as measured by Guest and FC versus the difference in FMH estimation between the two methods.

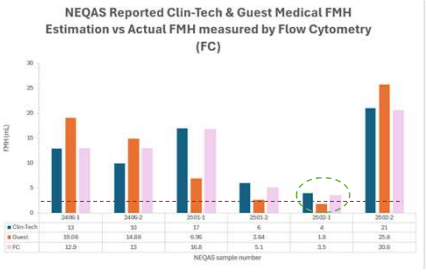

Figure 9 – Tri-method comparison of NEQAS samples for NHSBT referral. Difference between NEQAS FMH volumes when estimated using the Clin-Tech & Guest Medical Kleihauer methods and FC. The red dashed line indicates the Trust FC referral cut-off FMH volume. The green circle identifies a referral discrepancy between Clin-Tech and Guest, and the accurate FMH volume measured by FC.

		Clin-Tech	
		Standard prophylactic anti-D dose	Additional prophylactic anti-D dose
Guest	Standard prophylactic anti-D dose	12	6
	Additional prophylactic anti-D dose	6	16

Figure 10 – Clinical Impact Comparison. Al Green boxes = No difference in prophylactic anti-D required regardless of Kleihauer kit. Amber box = Guest kit potentially overexposes patients to prophylactic anti-D. Red box = Guest kit potentially results in insufficient prophylactic anti-D dosing and therefore high-risk situation.

Discussion

- Comparison of Guest and Clin-Tech demonstrated 100% agreement for the initial screen decision, essential for laboratory Kleihauer use (Figure 5).
- Bland-Altman analysis demonstrated acceptable agreement between Clin-Tech and Guest estimated slides, with 95% of results within 2SD with only 1 result being significantly different. The value is beyond the threshold for flow cytometry referral, so the patient impact is negligible. (Figure 6). Similar results were demonstrated with Guest FC Bland Altman (Figure 8) (Bland and Altman, 1986).
- Smaller SD from Guest-made slides indicated more reliability in the stain quality (Figure 7).
- A high-risk scenario was encountered in one NEQAS sample and the Guest Medical technique (Figure 9). Kleihauer FMH was estimated at 1.8mL so not triggering referral, but flow cytometry revealed 3.5mL FMH. But in the clinical environment under current Trust policy, sufficient prophylactic anti-D would have been given as the standard dose covers up to an 11mL FMH (Qureshi *et al.,* 2014).
- Considering Clin-Tech is a manual method and does not provide an unequivocally accurate FMH value, claiming successful validation solely based on agreement with a previously accepted technique is not robust. Comparison with the gold-standard FC provides a justification for successful validation claims and combined with acceptable agreement, is deemed valid by UKAS.

Conclusion:

The Guest Medical Kleihauer staining kit has the potential to replace the already established Clin-Tech staining kit despite the current study being underpowered to detect if the current statistically significant differences between the manual Guest kit and FC are constant. Furthermore, lack of realistic FMH volume samples hinders the generalizability of this study which is compounded by the small sample size under-representing borderline referral FMH volumes. The overarching and most important clinical aspect is that Guest can detect bleed volumes greater than the standard dose used at the Trust, minimising the potential impact on the quality of care provided to the mother and fetus. Subsequently, the Guest kit may be suitable to replace the Clin-Tech kit for Kleihauer testing in the Blood Transfusion laboratory.

Further Work

- Further validation is required to establish confidence in the Guest kit to consistently and appropriately identify borderline referral FMH volumes, mitigating the potential risk of insufficient prophylaxis (Figure 10).
- Reduce human input by increasing automation to standardize process – automated smearing and staining, and digital cell counting (Zhang *et al.,* 2021).

References