VALIDATION OF HAEMATOLOGY PERIPHERAL BLOOD SLIDE REVIEW RULES AT VARIOUS TIERS OF DIAGNOSTIC LABORATORIES IN SOUTH AFRICA

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DECLARATION

I, Nikki Bouwer, declare that this research report is my own, unaided work. It is being submitted for the degree of Master of Medicine in the branch of Haematology in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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N. Bouwer

..... Day of .....2017.

DEDICATION

This research report is dedicated to my family for their endless support during this journey and to all the staff working in the regional and district laboratories across South Africa facing increasing pressure in the workplace, may this study be one of many to lessen the load.

ABSTRACT

Automated analysers in the haematology laboratory have vastly improved since their inception in the mid-20th Century. The capability of these analysers to provide information exceeding a numerical output have made them allies in the clinical laboratory field. These analysers generate suspect flags and additional differential parameters with enhanced sensitivity and specificity which can assist in reducing manual peripheral blood smear (PBS) review rates and increasing laboratory productivity. In 2005, the staffing pressures faced by clinical laboratories prompted the International Consensus Group (ICGH) for Haematology Review to establish a set of criteria (consensus rules) to guide manual smear review of automated full blood counts (FBC) and white blood cell differential counts (Diff) in order to reduce unnecessary smear review rates. The usefulness of these rules is dependent on the patient population, laboratory criteria for PBS review and the specifications of the analyser.

The aim of this study was to apply ICGH rules to FBC samples (with and without Diffs) from tertiary, regional and district diagnostic laboratory tiers, to evaluate their efficiency, and to optimise their performance in the South African context.

In this study, the ICGH rules were compared to the individual representative laboratory standard operating procedures (SOP) for smear review in 600 samples from laboratories representing the tertiary, regional and district health tiers. Manual PBS review was the reference method. The rules were analysed for sensitivity, specificity and efficiency. False negative (FN) and false positive (FP) rates were also recorded for the rule set collectively, as well as for individual parameter and morphology flags. In order to optimise the ICGH rules for our setting, samples with FP flagging were then more closely analysed to determine if exclusion or modification of the flags triggered in these samples would be beneficial.

Smear review rates were substantially reduced on implementation of the ICGH rules in the tertiary laboratory as compared to the current laboratory SOP (82% vs 70%; p=0.022), were not

significantly altered in the regional laboratory (72% vs 80%; p = 0.198), while review rates increased on implementation of the rules in the district laboratory (67.1% vs 79.3%; p <0.0001). FN rates were reduced in all three sites, with the greatest reduction occurring in the district laboratory (dropping from 32% to 5%; p < 0.0001). Efficiency rates of the rule set were similar to those reported in the literature in the tertiary laboratory (78.1%), but were poorer in the regional (74.5%) and district laboratories (68.6%). Leukopenia and thrombocytopenia were the most common parameter flags triggered in the tertiary hospital (20.7% and 25% of samples respectively), while anaemia and microcytosis were most prevalent in the regional facility (22.5% and 17.5% of samples respectively). The most common abnormal parameter in the district laboratory was leucopenia (WCC<4 x 10^9/L) (16.4%). The analysers from the different tiers triggered morphology flags variably.

The findings of this study indicate the need for optimisation of the rules. Recommendations were made for each tier individually, each of which could reduce smear review rates further without increasing the clinically significant FN rates. In the tertiary laboratory, request for a Diff alone should not be a criterion for smear review and the white cell threshold for smear review should be reduced from 4 x 10^9/L to 2 x 10^9/L. Recommendations for the regional laboratory include modification of the WCC threshold to that of <2 x 10^9/L and exclusion of the platelet clumping (PCL) rule when the platelet count is normal/increased. In the district laboratory the nucleated red blood cell (NRBC) flag and PCL flag in samples with normal/high platelet counts should be excluded.

Conclusion:

Implementation of optimised ICGH rules would be beneficial to all laboratory tiers in South Africa, most particularly in the tertiary laboratory setting.

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ABREVIATIONS

ALYM	Atypical lymphocytes
ANISO	Anisocytosis
Diff	Differential count
DIM	Dimorphic blood picture
EDTA	Ethylenediamine tetra-acetic acid
ELLIP	Elliptocytes
FBC	Full blood count
FN	False negative
FP	False positive
FRAG	Red cell fragments
HB	Haemoglobin
HCT	Haematocrit
HCVAR	Variation in haemoglobin concentration
HJH	Helen Joseph Hospital
ICGH	International consensus group for haematology
IG	Immature granulocytes
Lab	Laboratory
LS	Left shift
LP	Large platelets
MACRO	Macrocytosis
MCHC	Mean cell haemoglobin concentration
MCV	Mean cell volume
MPV	Mean platelet volume
NHI	National health insurance
NHLS	National Health Laboratory Service
NRBC	Nucleated red blood cell
PBS	Peripheral blood smear

PC	Pencil cells
PCL	Platelet clumping
PLTS	Platelets
RBC	Red blood cell
RCC	Red cell count
RDW	Red cell distribution width
RETICS	Reticulocytes
RF	Rouleaux formation
SC	Sickle cell
SOP	Standard operating procedure
SPH	Spherocytes
TAT	Turnaround time
TC	Target cells
TD	Teardrops
TG	Toxic granulation
TN	True negative
TP	True positive
WBC	White blood cells
WCC	White cell count

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1. Background and rationale

1.1 History of the Full Blood Count

1.1.1 Manual Cell Counting and the Beginning of Automation

The interest in the inspection of blood in diagnostics dates back to ancient times where a very basic analysis included colour and structure of blood. A more detailed blood cell analysis became possible with the invention of the microscope more than 300 years ago and in 1852 the physiologist Karl Vierordt described the first counting method allowing for the quantitative analysis of the separate blood components (1, 2). In the late 1800's George Oliver proposed a new method of cell counting which was based on the visual perception of light loss due to absorption or scattering in a tube of diluted blood (3, 4). This method was improved by the addition of photodetectors with Mercandier *et al* showing in 1926 that a blood count could be derived if the photometer was adequately calibrated (4). In 1934 Moldavan proposed a method of counting cells as they flowed through a capillary tube on a microscope while in suspension (5). This method inspired Wallace and Joseph Coulter to introduce the world to the first non-optical cell counter at the National Electronics Conference in Chicago in 1956 (6, 7). This is referred to as the Coulter method.

1.2 Modern Analytical Principles

The principle of the Coulter method is also known as the "Electrical Sensing Zone" method (8). This method uses two electrodes, one placed in a tube with an aperture of a known size and the other in a low concentration electrolyte solution containing the particles of interest. An electrical field is applied creating an electrical current path. The impedance between the two electrodes is measured. As the particles pass through the aperture, the impedance changes and this is measured as a voltage pulse. The pulse height is proportional to the volume of electrolyte displaced. Cells are counted and sizes determined (7). The discovery of the Coulter principle for cell counting was

just the start for automated analysers in haematology as further improvements were on the horizon.

Later multi-parameter analysers capable of analysis by several methods such as optical, impedance and turbidimetry were developed. This allowed for improved precision, higher throughput and shorter turnaround time. A defining event in the transition from manual to automated cell counting came with the advent and improvement of high-throughput flow cytometry. This occurred in the 1960's and was refined in the 1970's to include fluorescence-based cell sorting (4, 9). Current haematology analysers make use of the following principles: Impedance (as is explained by the coulter principle), high frequency measurement, light scatter from different angles, fluorescence flow cytometry and spectrophotometry.

High frequency measurement is used predominantly to separate immature granulocytes from mature cells and usually occurs after red cell lysis. Impedance measurement and exposure to a high frequency current occur simultaneously. The result of this concurrent measurement and exposure determine the internal complexity of the cell therefore allowing for differentiation of cells (2, 10)

Light scatter measured at different angles is a method of measurement requiring a monochromatic light source (laser diode with a specific wavelength) which allows measurement of red blood cells (RBC), platelets (PLTS) and white blood cells (WBC). The light scatter is measured at different angles (11). Three main distribution curves are generated from the light scatter pattern of red cells and platelets including: mean cell volume (MCV) of RBC, mean platelet volume (MPV) and haemoglobin (HB) concentration (2).

Cytophotometry resulting in the measurement of cells using fluorescent labels attached to either membrane or cytoplasmic markers is now more commonly used in the immunological differentiation of WBC and cell counting for example differentiating B and T lymphocytes. The

principle is similar to that of scattered light except that fluorescent labelled cells are detected and the data processed to allow a visual representation of the different cells(12). In most instruments data is displayed as either histograms (usually for red cell, platelet and white cell relative numbers) or scatterplots (usually for identification of white cell subpopulations)(10).

Today modern analysers have a throughput of up to approximately 1000 full blood count analyses per hour.

These improvements allowed automation in the laboratory to play a more significant role, increasing accuracy, efficiency and productivity.

1.2.1 Analysers in the National Health Laboratory Service

The National Health Laboratory Services (NHLS) is the largest laboratory service provider in South Africa. The commonest analysers in use in the NHLS are the Siemens Advia 2120 (Siemens Healthcare Diagnostics, NY, USA), Sysmex family of analysers (XT2000i, XE, XN and XS) (Sysmex corporation, Kobe, Japan), and the Beckman Coulter range (Beckman Coulter, Inc, Brea, California, USA). The analytical principles of these instruments and the morphology flags generated by each of them are summarised in Table 1.1. Specifications of the different analysers are presented in Appendix A Table A.1.

Table 1.1 Analyser Principles

Analyser	Parameter	Principle
Advia 2120 (Siemens Healthcare Diagnostics, NY, USA)	**Parameter**	**Principle**
	Red cell count	Isovolumetric sphering Light scatter analysis
	Haemoglobin	Red cell lysis Colorimetric optical analysis
	Platelet count	Isovolumetric sphering Light scatter analysis (amplified 12-30x)
	White cell count & differential count:	**BASO chamber:** Red cells, platelets lysed Light scatter **PEROX chamber:** Red cells, platelets lysed White cell shrinkage Light scatter Peroxidase staining
	Morphology Flags	
	Large platelets	Refractive index between 1.35 & 1.40 Cell volume between 20fl and 60fl
	Platelet clumping	"Noise" area on PEROX cytogram
	Red cell fragments	Cell volume <30 but refractive index >1.40.
	Nucleated red blood cells (NRBCs)	Recognised on the BASO & PEROX channels according to size and nuclear density.
	Immature granulocytes (IG)	Large cells with high peroxidase activity. Seen in the neutrophil area of the PEROX cytogram and mononuclear area of the BASO cytogram.
	Blasts	Blast cluster of the BASO cytogram.
	Atypical lymphocytes	LUC region on PEROX cytogram.
Sysmex analysers (Sysmex corporation, Kobe, Japan)	**Parameter**	
	Red cell count	Hydrodynamic focusing and Impedance
	Haemoglobin	Red cell lysis Spectrophotometry
	Platelets	Impedance Optical fluorescence
	White cell count & differential count	Light scatter Fluorescence
	Morphology Flags	
	Immature granulocytes	IG cluster on the diff scattergram.
	Blasts	Blast cluster on diff scattergram.
	Left shift	Left shift cluster on diff scattergram.
	Atypical Lymphocytes	ALYM cluster on diff scattergram
	NRBC	? NRBC generated when clustered in NRBC region on diff scattergram.
	Lysis resistant RBC	Abnormal clustering in RBC ghost population region
	RBC agglutination	Calculated from RBC histogram. MCHC> 36.5g/dL
	Turbidity/HGB interference	Calculation and size comparison of MCV,MCHC,RDW
	Iron deficiency	Determined from reticulocyte scattergram
	Fragments	Clustering in right upper ghost regions in DIFF or NRBC scattergrams.
	Platelet clumps	Read from the PEROX scattergram. Not included in the diff count but recorded for flagging.

Table 1.1 continued

Analyser	Parameter	Principle
Beckman Coulter Act5Diff (Beckman Coulter, Inc, Brea, California, USA)	**Parameter**	
	Red cell count	Coulter principle
	Haemoglobin	Spectrophotometry
	Platelets	Coulter principle – differentiated from red cells according to size.
	White cell count/basophil count.	Coulter principle & differential lysis.
	Five part differential count	Flow impedance &cytochemical staining
	Morphology Flags	
	Large Immature cells	Larger volume and increased intensity of scattered light (if IMM reading is increased)
	Blasts	Larger than monocytes but similar absorbance. Some may be located between normal lymphocyte and monocyte populations.
	Small lymphocyte (SL)	Suspected abnormalities include small lymphocytes, platelet aggregates, NRBCs and lysis-resistant RBCs.
	Left shift	If increased number of cells in monocyte/neutrophils and neutrophil/lymphocyte gate on diff plot.
	Cold agglutinin	
	Schistocyte	MCHC value high
	Platelet aggregates	Derived from the platelet histogram
		Debris flag with low platelet count or debris with mean platelet volume >10 or debris with platelet distribution width >20.
	NRBC	
		If number of particles counted in the SL (small lymphocyte) or SL1 region of the diff plot of the analyser exceeds the set limit and the two WBC counts performed by the anlyser on the sample differ by more than the predetermined limit (i.e.WBC vote out) then this flag is triggered.

1.3 Instrument generated flags

Analysers use a range of techniques to detect abnormalities in the blood, be it quantitative (i.e. reflecting derangements of 1/more full blood count (FBC) parameter/s) or through the use of morphology flags. The latter vary somewhat between analysers, as their generation depends on the technology employed by the instrument in question. However, there are several morphology

flags which are common to most haematology analysers, including those querying the presence of platelet clumping (PCL), atypical lymphocytes (ALYM), blasts and immature granulocytes (IG). The instrument flags generated by the analysers commonly used in the NHLS are summarised in Table 1.1.

1.4 Advantages and Disadvantages of Automation

The benefits of using an automated analyser in haematology are numerous. These include speed, allowing a greater number of samples to be processed and results delivered to the clinician timeously. A result of these advantages is a reduction in technical staff required on the bench. Automated analysers assess in excess of 10,000 cells when determining FBCs, thus allowing for a high level of accuracy. Increased speed and accuracy when analysing routine samples allows for more time to be dedicated to verifying and further investigating abnormal results (13).

However, automated haematology analysers are not without challenges (14). Although the differential count of mature leucocytes is done with excellent precision and accuracy by most automated analysers, enumeration of immature leucocytes and assessment of both red cell and platelet morphology is still better performed manually.

Haematology analysers are renowned for being accurate in normal samples; however, there are situations in which these analysers provide artefactual results(14). Some of the reasons for this are highlighted in Table 1.2.

Table 1.2 Causes of spurious counts in haematology analysers

Cell Lineage	Parameter	Spurious result
Red blood cells (RBC)	Haemoglobin (Hb)	Lipaemia causing increased turbidity and hence falsely increased Hb results High white cell count causing turbidity Raised immunoglobulins may falsely elevate Hb result Cryoglobulins may prevent light transmission in Hb measurement, thus falsely increasing Hb results
	Red cell count (RCC)	Giant platelets may be interpreted as red cells in an impedance based counter Cold or warm agglutinins causing red cell clumps will result in lower red cell counts (RCC) and spurious mean cell haemoglobin concentration (MCHC) & haematocrit (HCT). Microcytic red cells may be counted as platelets Cryoglobulins may cause a flow anomaly interfering with the RCC.
White blood cells (WBC)		Neutrophil aggregation in vitro may cause decreased white cell count Platelet clumps and large platelets may mimic white cells Nucleated red blood cells may initially be included in the white cell count Cryoglobulins that cluster together may influence white cell count
Platelets (PLTS)		Ethylenediamine tetra-acetic acid (EDTA) may cause platelet clumping resulting in pseudothrombocytopenia. Platelet sattelitism may cause a falsely decreased platelet count Large platelets may be assessed as white cells or red cells Red cell fragmentation may falsely increase platelet count Micro-organisms may be interpreted as platelets

Manual determination of a differential count (Diff) and review of the PBS remains the gold standard, but is unfortunately time consuming, labour intensive and expensive and should be used

judiciously (15). In the face of qualified staff shortages, high sample volumes and turnaround time pressures, performing Diff counts and smear reviews manually undermines the efficiency and lowers the productivity of the laboratory.

1.5 Consensus guidelines

In 2005, the staffing pressures faced by clinical laboratories prompted the International Consensus Group for Haematology Review to establish a set of criteria (rules) to guide manual review of automated FBC and white blood cell Diff counts in order to reduce unnecessary smear review rates. The initial consensus rules were conceived and piloted in 17 laboratories in six countries (USA, Australia, Canada, United Kingdom, Spain and Switzerland). From this pilot, consensus was reached on which situations should trigger a manual review of automated counter results as well as further actions including further testing. The consensus rules were then validated in these same countries. Each laboratory was asked to test 1000 samples. A total of 13 298 patient samples were analysed on 6 pre-selected haematology analysers: Abbott CellDyn 4000 (Abbott Laboratories, Abbott Park, IL, USA), the ADVIA 120, the Beckman Coulter GenS and LH750 and the Sysmex SE-9000 and XE-2100. These analyses resulted in 41 rules for haematology review reached by consensus (see Appendix B). Of these 41 rules, 15 relate to the FBC parameters, 7 to the differential parameters, 2 to reticulocytes and 17 to red cell, platelet and white cell instrument suspect flags. The application of these rules in the validation arm of the study revealed a false negative (FN) rate of 2.9 % (16). Of interest in this study, although it took place in different laboratories using different analysers, all laboratories had similar truth tables (that is, true positives (TP), true negatives (TN), false positives (FP) and FN). Even having noted this, it is still vital that the rules be verified in each laboratory planning to implement them. There are a number of shortcomings and concerns with the ICGH consensus rules. For instance, the testing laboratories in the validation process were all in developed countries whose disease profile and available technology infrastructure is not the same as that seen in developing countries. These

rules were validated only in tertiary/referral laboratories but not throughout the whole spectrum of diagnostic laboratory services, and several subsequent reports evaluating the rule set have shown undesirably high FN rates (>5%)(17-20). In South Africa, very few studies evaluating the ICGH guidelines have been published, and all research in this regard to date has stemmed from academic centres. Local validation and optimisation of the rules in non-academic laboratories using a variety of analysers is thus required.

1.6 Clinically significant morphology

In the same year as the ICGH published the consensus rules for smear review, the New England Journal of Medicine published an article by Barbara Bain about the importance of peripheral blood smear (PBS) assessment (15). Certain clinical scenarios benefit greatly from examination of the PBS, however, the clinical setting of the samples that come into the laboratory are not always known to laboratory staff. There are, however, laboratory indices which should trigger an evaluation of the PBS in order to assist in providing the requesting doctor with a differential diagnosis and aid in further workup of their patients. The clinical disorders where PBS examination may provide such information include anaemia, thrombocytopenia/thrombocytosis and leukaemia, lymphoma and bone marrow failure. Within the context of anaemia the following clinically significant disorders may be detected using PBS review: haemolytic anaemia (including hereditary causes and the life-threatening microangiopathic haemolysis as well as haemolysis caused by oxidant damage) and megaloblastic anaemia. The value of PBS assessment in platelet count abnormalities lies in excluding pseudothrombocytopenia or falsely raised platelet counts as well as assessing possible causes including microangiopathic haemolysis, leukaemia/ lymphoma and malaria. It is imperative that cases of leukaemia or lymphoma be detected as early as possible and often the PBS evaluation is the first indication of such disorders (unexplained lymphocytosis, leucocytosis or monocytosis or a fortuitous finding) (15). All of these conditions present examples where examination of the PBS would provide vital information to the treating physician

and could be deemed clinically significant pathology. While this is not an exhaustive list of all clinically relevant pathology it may provide a minimum requirement of pathology that should not be missed when evaluating any rules for smear review used in a laboratory.

1.7 Aim & Objectives

Aim: To validate the ICGH rules for smear review in tertiary, regional and district NHLS laboratories and to optimise these rules for the South African disease burden**.**

Objective 1**:** To determine the manual review rate of blood smears at tertiary, regional and district laboratories in the NHLS before and after application of the ICGH rules.

Objective 2**:** To evaluate the false positive, false negative and efficiency rates before and after application of the ICGH rules.

Objective 3**:** To optimise the rules at tertiary, regional and district laboratories in the NHLS.

2. Materials and Methods

This study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (Appendix C).

2.1 Study Design

The study was a prospective, non-interventional cross-sectional study conducted over a 3 month period from 1st January to 31st March 2014.

2.2 Study Setting

The study was performed at health diagnostic laboratories from the tertiary, regional and district hospital tiers in the NHLS. Table 2.1 provides the diagnostic laboratory tier; the size of the hospital serviced and test volumes of the selected laboratories (as provided by the respective laboratory managers).

Table 2.1 Laboratory Demographics

	Helen Joseph Hospital	Tambo Memorial Hospital	Sebokeng Hospital	Dr Yusuf Dadoo Hospital
Tier	Tertiary	Regional	Regional	District
No. of beds	700	640	800	270
Monthly volume over study period	10180 FBCs &1061 Diffs	6222 FBCs & 584 Diffs	4256 FBCs & 827 Diffs	540 FBCs & 119 Diffs
No. of morphologists	2	2	7	2
Diff bench adequately staffed	No	Yes	No	Yes

These laboratories represent each tier in the South African health system as defined by the Regulation for the Designation of Public Hospitals laid out in the National Health Insurance (NHI) white paper released in December 2015 (21). Public hospitals are categorised into 5 tiers (Appendix D).

As a comprehensive study of the ICGH guidelines for smear review had already been performed at central hospital level (Charlotte Maxeke Johannesburg Academic Hospital) (22),the district, regional and tertiary laboratories were chosen to represent the diagnostic tiers.

The specific laboratories chosen were selected as they were representative of not only a wider area of Gauteng (East Rand (Tambo Memorial), West rand (Dr Yusuf Dadoo), South Gauteng (Sebokeng) and Central Gauteng (Helen Joseph)) but also represented the most commonly used analyser from each tier at the time this study was commenced. The Beckman Coulter Act5 Diff (Beckman Coulter Inc., California, USA), the Sysmex XT2000i (Sysmex, Kobe, Japan) and the Advia 2120 (Siemens healthcare diagnostics, NY, USA) were assessed in the district, regional and tertiary tiers respectively. Table A.1 in Appendix A summarises the three analysers assessed in this study.

2.3 Study Population and Eligibility Criteria

2.3.1 Inclusion and exclusion criteria

The peripheral blood samples used in the study were selected from the FBC and/or FBC and Diff samples analysed as part of the routine laboratory workload in the various hospital tiers. A request was submitted for 10 random samples to be selected each day over the three month period per laboratory. Samples were randomly selected in order to achieve a representative sample of each laboratory's patient population. Selection based on FBC counts or instrument generated

flags was discouraged in order to avoid selection bias. Exclusion criteria are summarised in Table 2.2.

Table 2.2 Exclusion Criteria

Exclusion Criteria
Inadequate staining of the PBS
EDTA or storage-related changes
Samples referred from clinics or sickbays with extended transport times (>48 hours)
Samples with inadequate volume (<4 ml)
Samples with a delay in reaching the respective laboratory where FBC/Diff was run
Results which indicated partial aspiration by the instrument

2.4 Data Collection

2.4.1 Assessment of manual review rates before implementation of the ICGH rules

In order to assess the manual review rates before implementation of the ICGH rules, the respective laboratory SOPs for smear review and abnormal FBC results were obtained. These SOPs were assessed to answer three questions. 1) What are the thresholds for smear review for the FBC and Diff count parameters? 2) Does the laboratory make use of morphology abnormalities flagged by the respective analysers? 3) If so, which flags do they use? The laboratory manager or pathologist in charge of the laboratory was also questioned on the operational requirements and client satisfaction that the current smear review practices provided. The aim of this line of questioning was to determine the operational need to implement a new system for smear review and whether a reduction in smear review rates was, in fact, required. The information collected included number of staff on the morphology bench, number of samples being requested for smear review and any specific concerns raised by the NHLS clients in view of FBC and Diff count results (See Appendix E).

2.4.2 Assessment of manual review rate after using the ICGH rules

The manual review rates after implementation of the ICGH rules were then assessed and compared to the manual review rate using the current laboratory SOP. Both assessments were performed on the same samples.

2.4.3 Laboratory Methods

Ten randomly selected FBC requests (with or without Diff counts) were sampled from each laboratory tier daily. The samples were collected into Ethylenediamine tetra-acetic acid (EDTA)-K_2 (Becton Dickinson, USA) blood tubes as per laboratory manual and run within 2 hours of sample collection where possible. Each sample was run as per laboratory SOP for standard FBC and Diff request. Daily quality controls were performed as well as external proficiency testing.

For each specimen a stained blood film (May-Grünwald Giemsa) was prepared for morphologic assessment. A printout was made of the accompanying counts, scatter plots (if available) and morphology flags. Manual peripheral blood smear (PBS) review was performed on all the films by an experienced technologist in each laboratory and subsequently reviewed by the investigator. Any smears not initially reviewed by the attending technologist were reviewed by Dr K Mannaru, a consultant haematologist.

Each sample was assessed using both the laboratory SOP and the ICGH rules and manual review rates compared. The indications for manual review were noted for each sample. A positive smear result was defined as per the ICGH criteria (see Table 2.3).

Table 2.3 Peripheral smear criteria (as per ICGH criteria)

Peripheral Smear Criteria
➢ RBC morphology at 2+/moderate or greater
➢ Malaria
➢ Giant platelets (PLTs) at 2+/moderate or greater
➢ PLT clumps at greater than 1+/mild
➢ Dohle bodies/toxic granulation/vacuoles at 2+/moderate or greater
➢ Blasts at 1 or greater
➢ Metamyelocytes at greater than 2
➢ Myelocytes/ promyelocytes at 1 or greater
➢ Atypical lymphocytes at greater than 5
➢ Nucleated red blood cells (NRBCs) at 1 or greater
➢ Plasma cell at 1 or greater

Each sample was allocated a research specific number in order to determine total number of samples and allowing for elimination of any unique patient identifying information.

The data was recorded on pre-designed data collection sheets (See Appendix F)

2.5 Data Analysis:

2.5.1 Analytical methods

Data was allotted in 2x2 truth tables and true/false positive, true/false negative and efficiency rates using the ICGH rules at each laboratory were assessed. A FN rate of <5% was set as threshold of acceptance (as recommended in the ICGH guidelines) (16). The sensitivity and specificity of each FBC parameter flag (white cell count (WCC) <4 or >30 x 10^9/L, HB <7g/dL or >2g/dL more than upper limit of normal, MCV<75 or >105 fl, red cell distribution width (RDW)>22% and platelets <100 or>1000 x 10^9/L) were determined for the detection of any

abnormality. Parameters within the same lineage were then combined (where possible) and the sensitivity and specificity for the detection of lineage specific abnormalities also calculated; i.e. samples with parameter rule violations related to the WCC were assessed for white cell morphology abnormalities, those with red cell parameter rule violations (HB/MCV and RDW) were assessed for red cell abnormalities, and those with platelet parameter rule violations were assessed for platelet abnormalities.

The sensitivity, specificity, FP and FN rates of the clinically significant analyser-generated morphology flags for each lineage were assessed in two ways:

- For the specific morphological abnormality flagged (e.g. normoblasts seen morphologically where normoblasts which were flagged).
- For morphologic abnormalities in the same cell lineage as the morphology flag (e.g. any red cell abnormalities where normoblasts were flagged).

The truth table information garnered from the flags that were triggered in 15 or fewer samples were analysed but the results were treated with reserve in view of the small sample size.

Samples with FP flagging were then more closely analysed in order to identify flags which trigger unnecessary review, thereby facilitating optimisation of the ICGH rules for the local setting.

For the flags commonly triggered in FP samples, close attention was paid to specimens where these flags were triggered in isolation so as to determine the effect of excluding these flags from the rule-set.

2.6 Statistical analysis

Data was captured on excel spreadsheet (Microsoft Office 2007, Redmond, USA) and analysed using GraphPad Prism(GraphPad Software, California, USA).

In the context of this study, the samples were defined as being true/false positive or negative as follows (16):

- True positive (TP): if a rule was triggered and this corresponded with a positive finding on the smear (for criteria for a positive smear see Table 2.4)
- False positive (FP): if a rule was triggered and a positive result was not found on smear review.
- False Negative (FN): if a rule was not triggered and a positive finding was noted on the smear.
- True negative (TN): if a rule was not triggered and no positive smear finding was detected.

All rates were calculated as a proportion of all included samples for each laboratory tier.

Sensitivity was calculated using the following formula: TP/ (TP+FN) x 100

Specificity calculated using the following formula: TN/ (TN+FP) x 100

The efficiency of each analyser was determined using the following formula:

Efficiency = (TP + TN)/All samples (TP+FP +TN+FN) x 100.

A Mann-Whitney U test was used to compare the manual review rates between different rule sets. A *P* value of <0.05 was considered statistically significant.

3. Results

3.1 Feedback from Laboratory Managers

The laboratory managers from the four laboratories which took part in this study gave their feedback with regards to staffing requirements and Department of Health complaints (Table 2.1). None of the laboratories reported having difficulties with Department of Health in terms of TAT complaints except when the laboratory LIS is not functioning optimally.

HJH, Tambo Memorial Hospital and Yusuf Dadoo Hospital all have two technologists competent in morphology. The haematology supervisor also aids in peripheral smear morphology at HJH when required.

3.2 Study population

A total of 600 samples were assessed from the three laboratory tiers (260 from the tertiary laboratory, 200 from the regional laboratories and 140 from the district laboratory).The pertinent demographic data and data by clinical division are summarised in Tables 3.1 and 3.2.

Table 3.1 Pertinent demographic data

	HJH (Tertiary)	Tambo Memorial/Sebokeng (Regional)	Dr Yusuf Dadoo (District)
Total samples (N)	260	200	140
FBC (N)	90	45	118
FBC/Diff (N)	170	155	22
Male (N)	110	94	68
Female (N)	140	101	72
Unspecified gender (N)	10	5	0
Median age in years [median(IQR)]	38.0 (27-53)	36.0 (20-48)	37.0 (31-45)

IQR: Interquartile range

Table 3.2 Data by clinical division

	HJH (Tertiary)	*Tambo Memorial/Sebokeng (Regional)*	*Dr Yusuf Dadoo (District)*
Medical ward (%)	40.7	18.0	26.4
Surgical ward (%)	4.6	19.0	7.9
Paediatric ward (%)	3.4	8.0	1.4
ARV clinic (%)	5.3	7.0	26.9
Outpatients (%)	9.6	9.0	15.3
ICU (%)	4.6	10.0	3.5
Admissions (%)	8.4	16.5	10.0
Emergency (%)	10.0	1.5	8.6
Not recorded (%)	13.4	11.0	-

3.3 Comparison of manual review rates between the current laboratory SOP and the ICGH rules

3.3.1 Manual review rates

The results of the manual review rates using the laboratory SOP versus implementation of the ICGH rules varied between laboratories depending on how restrictive the SOP rules were.

3.3.1.1 Advia 2120 [HJH]:

Two hundred and sixty samples were reviewed from the tertiary laboratory and cytopenias accounted for the majority of abnormal parameters, while samples with cytoses were few (Fig. 3.1). Implementation of the ICGH rules showed a statistically significant reduction in the manual review rate (82% vs 70%; p=0.022). The indications for smear review using the lab SOP included Diff count requested by the physician (171/260; 66%) and thrombocytopenia (67/260; 26%) while the indications for smear review using the ICGH rules included analyser morphology flags

(172/260; 67%), WCC <4 x 10^9/L (8/260; 20%), WCC >30 x 10^9/L (5/260; 2%), Hb <7g/dL (29/260; 11%), Hb >2+ upper limit (5/260; 2%), PLTS <100 x 10^9/L (68/260; 26%), MCV <75 fl (16/260; 6%), MCV >105 fl (21/260; 8%) and RDW >22% (16/260; 6%).

3.3.1.2 Sysmex XT2000i [Tambo Memorial/Sebokeng]:

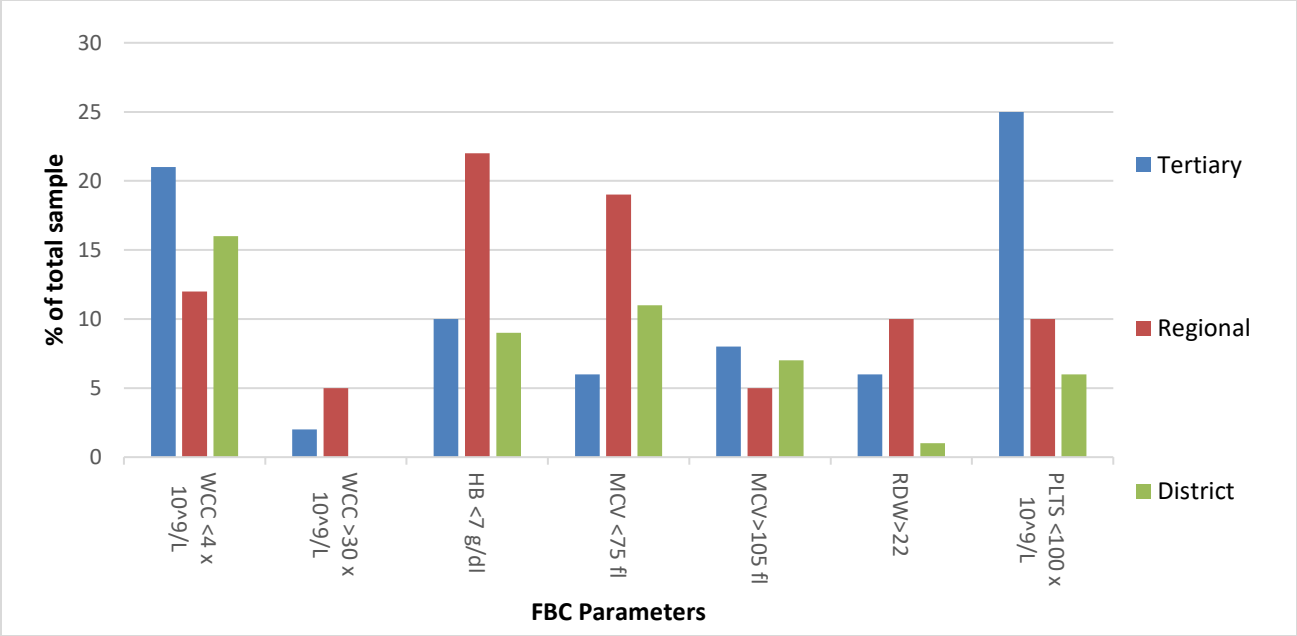
Two hundred samples were obtained from the regional laboratories, where the SOP used was based broadly on the ICGH rules with some adjustments. There was no available documentation regarding the validation of these rules. There was no significant increase in manual review rate in the regional tier laboratory (72% vs 80%; p = 0.198). The indications for smear review included parameter abnormalities for both the lab SOP and ICGH rules. The main difference between the two groups was the addition of morphology flag assessment in the ICGH cohort which accounted for 68% of the samples manually reviewed.

3.3.1.3 Beckman Coulter Act5diff [Dr Yusuf Dadoo]:

One hundred and forty samples were obtained from the district laboratory, where the SOP used was similar to that used in the regional laboratory. Significantly more slides were manually reviewed when the ICGH rules were applied (67% vs 79%; p <0.0001). The indications for smear review were similar in both groups with the primary difference being the WCC<4 x 10^9/L used by ICGH versus WCC <2 x 10^9/L used in the lab SOP.

3.4 Comparison of analyser flags and morphology findings

The proportion of FBC parameters and morphology flags which triggered peripheral smear review in the sample populations of the different diagnostic tiers are depicted in Figures 3.1 and 3.2 respectively.

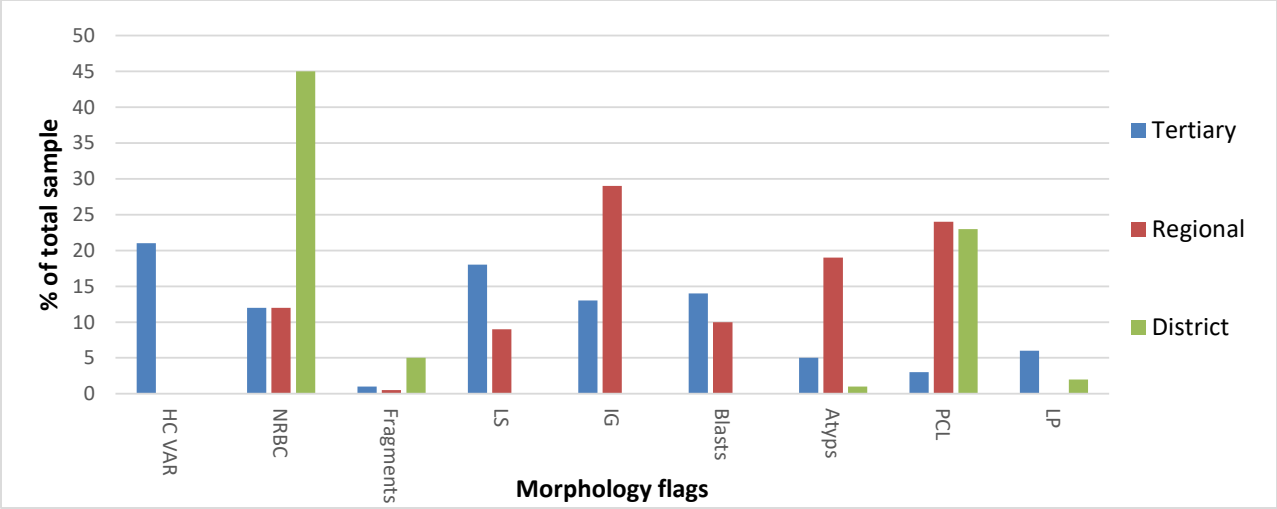


(WCC: white cell count; HB: Haemoglobin; MCV: mean cell volume; PLTS: platelets).

Figure 1 Proportion of abnormal parameters as a percentage of all samples per tier.

Leukopenia and thrombocytopenia were the most common parameter flags triggered in the tertiary hospital, while anaemia and microcytosis were most prevalent in the regional facility (Fig. 1). The most common abnormal parameter in the district laboratory was leucopenia (WCC<4 x 10^9/L), with <10% of samples having anaemia or thrombocytopenia.

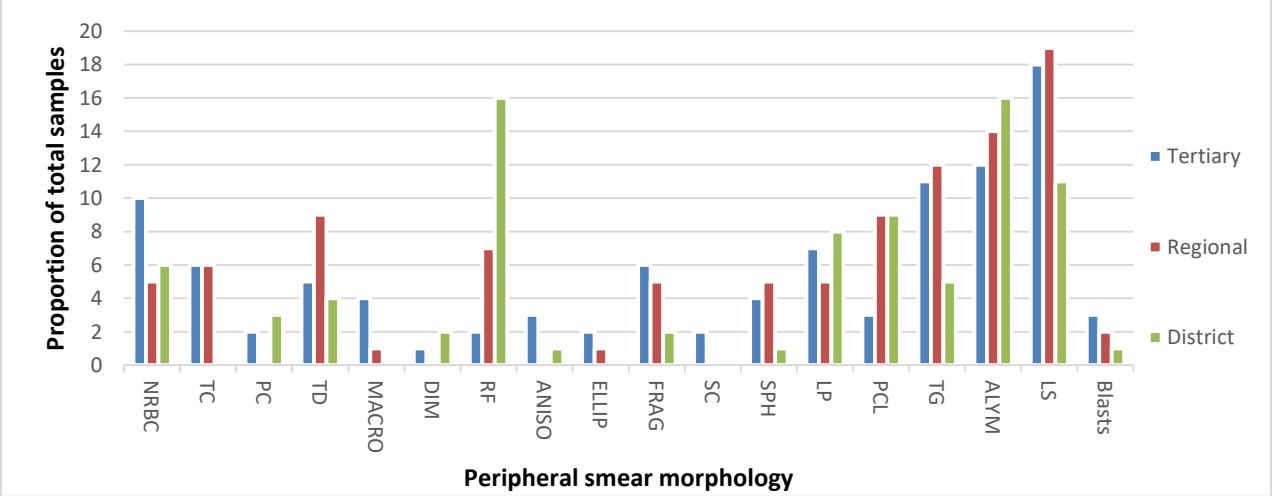
The analysers from the different tiers triggered the different morphology flags variably (Fig 2) based on the sample population collected at the respective laboratories and instrument generated flags specific to each analyser.



(HC VAR:variation in haemoglobin concentration, NRBC: nucleated red blood cells, LS: left shift, IG: immature granulocytes, ALYM: atypical lymphocytes, PCL: platelet clumping

Figure 2 Proportion of individual flags as a percentage of total flags per tier

The majority of peripheral blood morphology abnormalities appear to mirror one another in the three tiers with the exception of a few cases (Fig. 3).



(NRBC: nucleated red blood cells, TC: target cells, PC: pencil cells, TD: teardrops, MACRO: macrocytosis, DIM: dimorphic blood picture, RF: rouleaux formation, ANISO: anisocytosis, ELLIP: elliptocytes, FRAG: red cell fragments, SC: sickle cells, SPH: spherocytes, LP: large platelets, PCL: platelet clumping, TG: toxic granulation, ALYM: atypical lymphocytes, LS: left shift)

Figure 3 Commonest peripheral smear findings from each laboratory tier as a proportion of all samples assessed.

Table 3.3 summarises the most pertinent morphology findings from each diagnostic tier.

Table 3.3 Peripheral blood morphology in different laboratory tiers

Tertiary tier [HJH]:	Regional tier [Tambo Memorial/ Sebokeng]:	District tier [Dr Yusuf Dadoo]:
Greater number of smears with NRBCs were noted	Samples with teardrops, LS and toxic granulation were more commonly seen.	RF, ALYM, LS and PCL most commonly noted. NRBCs were seen in only 6% of samples, despite the analyser having flagged for this abnormality in ~45% of the included specimens.

3.5 Truth tables and false negative/positive analyses

3.5.1 Tertiary laboratory: Helen Joseph Hospital (Advia 2120)

Table 3.4 summarises the changes in FN rate, FP rate, sensitivity, specificity and efficiency between the laboratory SOP and implementation of the ICGH rules.

Table 3.4 Truth table comparing laboratory SOP with ICGH rules

	Laboratory SOP	ICGH Rules
False negative rate [n/N(%)]	31/260 (11.9)	15/260 (5.8)
False positive rate [n/N(%)]	81/260 (31.2)	40/260 (15.4)
Sensitivity (%)	80.8	89.4
Specificity (%)	17.3	59.6
Efficiency (%)	56.9	78.1

Tables 3.5-3.7 are the truth tables for morphology flags, general lineage parameter flags and individual parameter flags respectively. These tables summarise the best and worst performing individual flags for both morphology and parameters.

Table 3.5 Tertiary laboratory morphology truth table

	Sensitivity (%)	Specificity (%)	Efficiency (%)	False Negative (%)	False Positive (%)
HC VAR for RBC	43	87	75	17	10
NRBC for NRBC	30	90	83	9	9
NRBC for RBC	25	94	72	24	4
FRAGS for fragments	-	-	-	-	-
Left shift for LS	47	92	82	12	6
Left shift for WBC	40	94	82	19	4
Immature granulocytes for IG	40	96	83	13	3
Immature granulocytes for WBC	32	98	77	22	2
Blasts for blasts	45	88	86	2	12
Blasts for WBC	23	91	70	25	6
Atypical Lymphocytes	13	97	84	13	2
Large platelets for LP	25	96	90	7	4
Large platelets for PLTS	21	97	88	10	3
Platelet clumping for PCL	40	98	96	2	2
Platelet clumping for PLTS	12	98	87	12	2

Table 3.6 Parameter truth table per lineage

Lineage	Sensitivity (%)	Specificity (%)	Efficiency (%)	False Negative (%)	False Positive (%)
White cells	26	81	58	30	12
Red cells	52	87	76	15	9
Platelets	34	76	71	83	21

Each parameter was assessed with regard to morphologic abnormalities in their specific cell line i.e. white cell abnormalities assessed in those samples with white cell parameter abnormalities.

Table 3.7 Individual parameter truth table

	Sensitivity (%)	Specificity (%)	Efficiency (%)	False Negative (%)	False Positive (%)
White cells count <4 x 10^9/L	23	81	56	43	11
White cell count>30 x 10^9/L	3	99	58	43	0.5
Haemoglobin<7g/dl	20	93	72	44	5
Haemoglobin >2 of upper limit	3	98	69	67	2
MCV<75fl	16	98	73	19	1
MCV>105fl	16	97	71	30	2
RDW>22	16	98	73	19	1
Platelets<100 x 10^9/l	34	76	71	83	21
Platelets >1000 x 10^9/L	-	-	-	-	-

3.5.1.2 Analysis of samples with false positive flagging

Because FP flagging results in increased manual review rates, flagged samples with no morphological abnormalities were assessed in order to identify flags which trigger unnecessary review most frequently in normal samples. From the 40 samples that were assessed to FP using the ICGH rules, 25/40 (62.5%) were owing to morphology flags being triggered (Table 3.8).

Table 3.8 Analysis of false positive samples at the tertiary site

Flag triggered in false positive sample	Number of samples in which flag was triggered (N=40) [n (%)]	Triggered in isolation (i.e. no other morphology or parameter abnormality triggered) (Yes/total)
HCVAR	11/40 (27.5)	3/11
NRBC	4/40 (10.0)	1/4
Left shift	6/40 (15.0)	4/6
Immature granulocytes	1/40 (2.5)	0/1
Blasts	3/40 (7.5)	0/3
Atypical lymphocytes	1/40 (2.5)	0/1
Large platelets	3/40 (7.5)	0/3

The most commonly triggered flags in samples with FP review were the haemoglobin concentration variation (HCVAR), left shift (LS) and WCC <4 x 10^9/L flags (the latter particularly in samples with a WCC from 2-3.99x10^9/l). The HCVAR flag was flagged in isolation in only three samples (n=11, 27.2%) while the LS flag was triggered in isolation in four samples (n=6, 66.7%).

The HCVAR flag was triggered in 42 samples assessed as being TP. In four of these (n=42, 11%) the HCVAR flag was triggered in isolation. The morphology findings noted in these four samples included; a leukoerythroblastic reaction (with moderate LS and toxic granulation (TG)), spherocytes, ALYMs>5, and moderate target cells (TC) with nucleated red blood cells (NRBCs) (4/100 WBC) respectively. Out of all samples assessed, the HCVAR flag was triggered in isolation in nine (n=260, 3.4%).

The LS flag was triggered in 44 samples, 38 of which were TP (n= 44, 84%). Four of these samples had LS in isolation. A single sample which flagged LS, demonstrated 1% blasts with toxic changes (LS and TG), Blasts or ALYMs were not flagged by the analyser. In view of the

morphology findings, ongoing monitoring of the counts and PBS review was indicated. As a proportion of all tertiary laboratory samples, 8 (n=260, 3%) had a LS flag in isolation.

The WCC was 2-3.99 x 10^9/L in 48 samples, of which 32 (n=48, 66.7%) were TP. This flag triggered in isolation in 10 instances, of which two (n=10, 20%) were TP. The morphology in these samples included TG, ALYMs, rouleaux formation (RF), TC, large platelets and PCL with a platelet count <100x10^9/L.

3.4.2 Regional Laboratory Results: Tambo Memorial & Sebokeng Hospital (SysmexXT2000i)

Table 3.9 summarises the FN, FP, sensitivity, specificity and efficiency of the laboratory SOP versus implementation of the ICGH rules, showing an improvement in FN rate and sensitivity but at the expense of an increased FP rate and reduced efficiency.

Table 3.9 Truth table comparing laboratory SOP with ICGH rules

	Laboratory SOP	ICGH Rules
False negative rate [n/N(%)]	11/200 (5.0)	7/200 (3.5)
False positive rate [n/N(%)]	35/200 (17.5)	43/200 (21.5)
Sensitivity (%)	90.8	94.2
Specificity (%)	56.3	44.9
Efficiency (%)	77.0	74.5

Tables 3.10 – 3.12 summarise the truth tables for morphology flags, general lineage parameter flags and individual parameter flags for the regional tier laboratories and highlight the best and worst performing individual flags.

The sensitivity of the morphology flags was overall poor for both general and specific morphology findings but marginally better for the specific morphology (Table 3.10).

Table 3.10 Regional laboratory truth table

	Sensitivity (%)	Specificity (%)	Efficiency (%)	False Negative (%)	False Positive (%)
NRBC for NRBC (%)	30	89	87	3	11
NRBC for RBC (%)	22	92	73	21	6
Left shift for LS (%)	24	95	82	14	4
Left shift for WBC (%)	22	96	77	20	3
Immature granulocytes for IG (%)	71	82	78	5	15
Immature granulocytes for WBC (%)	63	84	77	9	12
Blasts for blasts (%)	33	91	90	1	8
Blasts for WBC (%)	17	93	75	20	5
Atypical Lymphocytes (%)	56	87	82	6	11
Atypical lymphocytes for WBC (%)	30	87	68	23	9
Platelet clumping for PCL (%)	61	80	79	4	18
Platelet clumping for PLTS (%)	54	81	78	6	17

Table 3.11 Parameter flag truth table

	Sensitivity	Specificity	Efficiency	False Negative	False Positive
White cells [%]	21	86	66	25	10
Red cells [%]	57	67	67	13	24
Platelets [%]	15	91	81	11	8

Each parameter was assessed with regard to morphologic abnormalities in their specific cell line i.e. white cell abnormalities assessed in those samples with white cell parameter abnormalities

Table 3.12 Individual parameter flag truth table for the regional tier laboratories

	Sensitivity	Specificity	Efficiency	False Negative	False Positive
White cells count <4 x 10^9/l [%]	9	87	62	30	9
White cells count >30 x 10^9/l [%]	9	99	69	30	1
Haemoglobin<7g/dl [%]	34	82	69	18	14
Haemoglobin>2g/dL of upper limit [%]	-	-	-	-	-
MCV<75fl [%]	28	87	70	21	10
MCV>105fl [%]	7	96	76	26	3
RDW>22 [%]	29	97	78	20	2
Platelets<100 x 10^9/l [%]	15	91	81	11	8
Platelets>1000x 10^9/l [%]	-	-	-	-	-

3.4.2.2 Analysis of samples with false positive flagging

From the 44 samples that were assessed to be FP using the ICGH rules (Table 3.13), 30 (n=44, 68.2%) were owing to morphology flags being triggered, 8 (n=44, 18.2%) were attributable to the WCC<4 x 10^9/L parameter rule (all of which were triggered in samples with a WCC 2-3.99 x 10^9/l) and 6 (n=44, 13.6%) were attributable to Hb<7 g/dL.

Table 3.13 Analysis of false positive samples at the regional sites

Flag triggered in false positive sample	Number of samples in which flag was triggered (N=44) [N (%)]	Triggered in isolation (i.e. no other morphology or parameter abnormality triggered) (Yes/total)
NRBC	2 (4.4)	0/2
Left shift	1 (2.2)	0/1
Blasts	3 (6.7)	0/3
Atypical lymphocytes	9 (20.0)	2/9
Immature granulocytes	9 (20.0)	4/9
Platelet clumping	13 (28.9)	4/13

The flags most frequently triggered in FP samples, the ALYM, IG, PCL and WCC 2-3.99 x 10^9/L flags were analysed further. The ALYM flag was triggered in 38 samples, of which 29 (n=38; 76.3%) were TP. This flag triggered alone in five instances (n=38; 13.2%), of which three were regarded as TP. All of these samples had ALYMs reported morphologically.

The IG flag was triggered in 58 samples of which 48 (n=58; 82.7%) were TP and 10 were triggered in isolation. Six of the latter samples (n = 10; 60%) were regarded as TP. The morphology findings included: one sample with a morphological finding of blasts (<20% of total white cells), while the others showed features of infection (LS and/or TG) and ALYMs.

The PCL flag triggered in 47 samples, and was TP in 34 (n = 47; 72.3%) of these. It triggered in isolation in six cases, two of which were TP as PCL was detected. In the TP cases, both of these samples had normal platelet counts and the finding was not clinically significant.

The WCC 2-3.99 x 10^9/L flag triggered in 20 samples, of which 11 (n=20, 55.0%) were TP. This flag triggered in isolation in 4 instances, all of which were FP.

The Hb<7g/dL flag was triggered in 45 samples of which 5 (n=45, 11.1%) were triggered in isolation. Four of these (n=5, 80%) were assessed as being FP with one TP sample (n=5, 20%). The TP sample had a Hb of 5.0g/dL with a morphological finding of TG.

3.4.3 District Laboratory Results: Dr Yusuf Dadoo Hospital (Beckman Coulter)

The changes noted on implementation of the ICGH rules in the district laboratory are summarised in Table 3.14.

Table 3.14 Truth table comparing laboratory SOP with ICGH rules

	Laboratory SOP	ICGH Rules
False negative rate [n/N(%)]	45/140 (32.1)	7/140 (5.0)
False positive rate [n/N(%)]	9/140 (6.4)	37/140 (26.4)
Sensitivity (%)	43.8	91.4
Specificity (%)	84.7	37.3
Efficiency (%)	60.7	68.6

Tables 3.15 – 3.17 summarise the truth tables for the district tier laboratory. These tables include morphology flags, general parameter flags and individual parameter flags respectively, highlighting the best and worst performing flags.

Table 3.15 District laboratory morphology truth table

	Sensitivity	Specificity	Efficiency	False Negative	False Positive
NRBC for NRBC (%)	66	39	36	1	52
NRBC for RBC (%)	58	38	37	7	45
Fragments for Fragments (%)	66	96	94	1	4
Fragments for RBC (%)	11	97	74	24	2
Left shift for LS (%)	-	-	-	-	-
Left shift for WBC (%)	-	-	-	-	-
Immature granulocytes for IG (%)	-	-	-	-	-
Immature granulocytes for WBC (%)	-	-	-	-	-
Blasts for blasts (%)	-	-	-	-	-
Blasts for WBC (%)	-	-	-	-	-
Atypical Lymphocytes (%)	-	-	-	-	-
Atypical lymphocytes for WBC (%)	-	-	-	-	-
Platelet clumping for PCL (%)	57	81	78	4	17
Platelet clumping for PLTS (%)	58	84	79	7	13

Table 3.16 Parameter flag truth table

	Sensitivity	Specificity	Efficiency	False Negative	False Positive
White cells [%]	26	87	69	23	9
Red cells [%]	46	83	73	14	13
Platelets [%]	17	96	83	14	4

Each parameter was assessed with regard to morphologic abnormalities in their specific cell line i.e. white cell abnormalities assessed in those samples with white cell parameter abnormalities

Table 3.17 District laboratory parameter truth table

	Sensitivity	Specificity	Efficiency	False Negative	False Positive
White cells count <4 x 10^9/l [%]	26	87	69	23	9
White cells count >30 x 10^9/l [%]	-	-	-	-	-
Haemoglobin<7g/dl [%]	26	98	78	21	1
Haemoglobin<2g/dL of upper limit [%]	-	-	-	-	-
MCV<75fl [%]	24	94	74	22	4
MCV>105fl [%]	3	91	66	27	6
RDW>22 [%]	-	-	-	-	-
Platelets<100 x 10^9/l [%]	17	96	83	14	4
Platelets<100 x 10^9/l [%]	-	-	-	-	-

3.4.3.2 Analysis of samples with false positive flagging

From the 37 samples that were assessed to be FP using the ICGH rules, 34 were owing to morphology flags being triggered (Table 3.18).

Table 3.18 Analysis of false positive samples

Flag triggered in false positive sample	Number of samples in which flag was triggered (N=37) [N (%)])	Triggered in isolation (i.e. no other morphology or parameter abnormality triggered) (Yes/total)
NRBC	31 (83.8)	22/31
Platelet clumping	3 (8.1)	0/3

The only flags triggered as FP were the NRBC and PCL flags. The latter was never triggered in isolation and all three samples had platelet counts >100 x 10^9/L. The other flags triggered in

conjunction with the PCL flag included WCC < 4 x 10^9/L (in 2/3) and NRBC flag (in all three). These samples would, therefore, have been manually assessed for another reason.

The WCC <4 x 10^9/L flag triggered in 23 samples, of which 19 (n=23, 82.6%) were TP. This flag triggered in isolation in 4 instances. This included 3 samples assessed as TP and one FP sample. The morphology detected included RF in three samples and ALYMs in one.

4. Discussion

In an effort to standardise the criteria for smear review across laboratories in all the health tiers in the Johannesburg area, the ICGH rules for smear review were validated using a cross sectional representative sample of FBC and Diff count analyses. Six hundred FBC samples with or without an accompanying Diff count were used in the validation of the ICGH rules and compared to the current laboratory SOPs. The manual review rates, FN and FP rates were evaluated and from these results sensitivity, specificity and efficiency were calculated.

4.1 Manual review rates

The implementation of rules for smear review resulted in a reduction of manual PBS review rates across all diagnostic health laboratory tiers in the sample of laboratories examined. The laboratories assessed in this study all had SOPs outlining the rules for smear review used, but none had clear evidence for the chosen approach nor was there a clearly documented validation of these rules. Of concern was the lack of standardisation of the rules determining manual smear review between the different diagnostic laboratories which only served to highlight the need for investigation into one standardised approach for all laboratories within a laboratory tier and an analyser-specific optimisation protocol based on validation results.

In the tertiary laboratory (HJH), 66% of smear reviews were performed in samples which had a Diff count requested, with a further 27% of the samples being manually reviewed because of thrombocytopenia (Plts $<140 \times 10^9$/L). Implementation of the ICGH rules in this sample population saw a statistically significant decline in the number of slides manually reviewed (from 82% to 70%; p=0.02). It should be noted that in the daily functioning of the laboratory ~10% of the total FBC requests have an accompanying Diff count request as compared with 66% in this study population. The smear review rates reflected here using the current laboratory SOP are thus likely to be inflated. Nonetheless, these findings support that a Diff count request alone need not

be a criterion for manual review of the peripheral smear. A similar recommendation was made by the Francophone group, who suggested that physician opinion alone not be a criterion for smear review except in haematology-oncology patients (20).

The regional tier laboratories (Tambo Memorial Hospital and Sebokeng Hospital) use a SOP based on the modified ICGH rules with some adjustments, however, it was unclear to what extent these rules had been validated in these laboratories. The manual review rate increased marginally in the regional laboratory but this was not statistically significant. The main reason for this was the inclusion of analyser morphology flags as criteria for slide review (which were not used in the laboratory SOP). A WCC of $<4 \times 10^9$/L as the threshold for smear review versus 2×10^9/L in the laboratory SOP also contributed to the increased review rate. As the majority of samples came from patients of African descent (who are known to have lower WCCs (23)), an argument could be made for the reduction of this cut-off to reduce unnecessary smear review.

The district laboratory (Dr Yusuf Dadoo) used the same SOP as the regional laboratory with the addition of morphology flags. There was a statistically significantly increase in the review rate with implementation of the ICGH rules in this tier (79.3% vs 67.1%; $p < 0.0001$). The main contributor to this difference was the stricter WCC threshold (4×10^9/L vs 2×10^9/L).

The review rates noted in the current study using the ICGH rules in all three tiers (70.0% in the tertiary laboratory, 79.5% in the regional laboratory and 79.2% in the district laboratory) are higher than those documented in several other studies evaluating the ICGH rule set. Eldanasoury *et al* from Egypt compared the ICGH criteria for smear review to their current laboratory smear review criteria and found that the review rate dropped from 71% to 54.25% on implementation of the ICGH rules (17). On implementation of the ICGH rules by Comar *et al* from Brazil, the manual review rate was reduced to 46.03%(18). Pratumvinit *et al* in Thailand compared the ICGH rules to their laboratory criteria and then to optimised criteria and found manual review rates of 29.33% using the ICGH consensus rules. (24). In their comparison study of the rates of

manual peripheral blood smear review using the Advia 2120 (Siemens Healthcare Diagnostics, NY, USA), UnicelDxH 800 (Beckman Coulter, Inc, Brea, California, USA) and SysmexXE2100 (Sysmex corporation, Kobe, Japan), Kim *et al* found manual review rates of 20.2%, 22.8% and 28.6% respectively (19). The reason for this discrepancy of review rates with the published literature may be population-specific, reflecting genetic differences among South Africans or a different pathology profile (particularly a higher burden of infectious diseases due to the HIV epidemic). Comparison of implementation of the ICGH consensus rules with other Sub-Saharan African countries was not possible as similar studies have not been published. The closest comparison is with a study done in South Africa by Joubert *et al*(25), who looked at the inbuilt flagging capability of the Sysmex XT-series analysers and found "positive" flagging in 63.7% of samples, a number much closer to those seen in this study. Optimisation of the rules in the South African setting is clearly required to reduce these rates in light of the shortage of staff skilled in morphology. Possibly, the criteria used for defining positive smear findings should be re-evaluated to take into account our unique population, pathology and labour constraints, so that morphology findings which are not clinically critical are given less weight when determining the FN rates, and validating/optimising smear review rules.

In the South African context of high workload owing to increased burden of infectious disease eg. HIV and Tuberculosis (TB), rules that reduce the manual PBS review rate, particularly in the more scarcely staffed regional and district laboratories, would allow the small complement of staff to focus on the truly pathological samples and provide a better diagnostic service.

4.2 Parameter evaluation

A greater proportion of samples with cytopenias (146/260, 56%) were received from the tertiary tier laboratory. This is an intuitive finding as one would expect patients at this hospital to have more severe cytopenias, being a referral hospital for both regional and district hospitals. A greater proportion of samples triggering parameter flags would require a greater number of staff

competent in morphology which was not the case in this study as the tertiary laboratory had the same number of morphologists as the district laboratory and fewer than the regional laboratory.

4.2.1 Tertiary tier laboratory (HJH)

The sensitivity for the parameter flags was poor, with all but the flag for a platelet count <100x10^9/l having a sensitivity >30%. (Table 3.7) The FN rate for the parameter flags was also extremely poor, being ≥ 30% in all but the flag for a MCV<75fl or a RDW >22.

When the parameter flags were assessed per lineage (and not per parameter), collectively the red cell, white cell and platelet lineage parameter flags did not show much improvement when reviewed in conjunction with general morphology findings per cell lineage, with only the combined red cell flags demonstrating a sensitivity >50% (Table 3.6). The FN rate for the parameter flags was also extremely poor, being ≥ 30% in all but the red cell flags (Table 3.6). Overall efficiency was poorest in the white cell flags, largely due to flagging in samples with a WCC< 4 x 10^9/L. This was particularly true among those with a WCC from 2-3.99x10^9/l, with 16 (n=48, 33.3%) cases with a WCC in this range being FP. However, when the rules were applied collectively (over all lineages in all samples), the sensitivity of the ICGH rules approached 90%, with a FN rate only marginally above the recommended threshold of 5% owing to the small number of pathological samples in each subgroup (Table 3.4).

4.2.2 Regional tier laboratories (Tambo Memorial/Sebokeng)

As was seen in the tertiary laboratory, the sensitivity of the parameters was poor, particularly for the white cell and platelet flags. The only parameter with a sensitivity >30% was Hb<7g/dL (Table 3.12). The WCC<4x10^9/l had the poorest efficiency rate (62%), with 8 (n=20, 40%) of those with a WCC from 2-3.99x10^9/l being FP samples.

When the lineage parameters were combined, the specificity was excellent for white cell and platelet parameters and still >60% for red cell parameters (Table 3.11). Red cell parameters were

the only lineage with a sensitivity >50% (57%) but platelet parameters had the best overall efficiency (81%), FN rate (11%) and FP rate (8%).

4.2.3 District tier laboratory (Dr Yusuf Dadoo)

As was seen in the tertiary and regional laboratories, the sensitivity of the parameters was very poor (See Table 3.17) when assessed as a single parameter. The specificity was excellent for all parameters. Efficiency was >60% for all the parameters, but poorest for the MCV>105 fl and WCC <4 x 10^9/L flags (66% and 69% respectively).

When the parameter flags were assessed per lineage (and not per individual parameter), collectively the red cell lineage had the best sensitivity with both white cell and platelet parameters achieving a sensitivity of <30%. The specificity and efficiency of all lineages was good though, however the FN rates were undesirably high (Table 3.16).

4.3 Morphology flags

A direct comparison between the morphology flags from the different laboratory tiers could not be done as the analysers used in the three tiers were not the same. Furthermore, the samples included were not analysed on more than one analyser, and differences between the morphology flags triggered by different analysers in the same sample has thus not been assessed. Nonetheless, there were considerable differences in the flags triggered by the analysers from the different tiers, even in those flags triggered by all three analysers. Of note, the HCVAR, LS and blast flags were the most common flags triggered by the Advia (tertiary tier laboratory) while the Sysmex analyser (regional tier laboratory) flagged a higher proportion of IG, ALYMs and PCL. NRBCs and PCL were the most commonly triggered flags by the Beckman Coulter (district laboratory) analyser.

4.3.1 Tertiary tier laboratory (HJH)

The analyser-generated morphology flags were assessed for sensitivity, specificity, efficiency, FN and FP rates for both the specific morphology finding for which they were flagged e.g. red cell fragments as well as general morphology findings in the lineage specified (i.e. Red cells, white cells or platelets).

Analysis revealed generally low sensitivity but high specificity for both general and specific morphology findings (Table 3.5). No morphology flag had sensitivity>50% and most had FN rates in excess of 10%. FP rates were generally low.

4.3.2 Regional tier laboratories (Tambo Memorial/Sebokeng)

Analysis revealed sensitivity exceeding 50% for the ALYM, IGs and PCL flags in both general and specific morphology analysis. Specificity was >80% for all morphology flags and efficiency rates were >70%, meaning they may be useful in guiding PBS review. The FN rates for most of the morphology flags were good when assessed for the specific finding with the exception of the LS flag. In comparison, the FN results for the general lineage morphology was>20% in all but the IG and PCL flags (Table 3.10).

4.3.3 District tier laboratory (Dr Yusuf Dadoo)

Analysis revealed good sensitivity for specific morphology findings in the flags triggered by the Beckman Coulter in this sample population. Sensitivity for the more general morphology was good for all flags other than the fragment flag. Specificity was good for the fragment and PCL flags but very poor for the NRBC flag. This translated to very good efficiency for the fragment and PCL flags for both general and specific morphology but again, poor efficiency of the NRBC flag. The FN rates for most of the morphology flags were good when assessed for the specific

finding and not as good for general lineage morphology (Table 3.15). This was particularly true for the fragment flag's general morphology.

Eldanasoury *et al* from Egypt using the Beckman Coulter LH750 on 800 samples also found the NRBC and PCL flags to be two of the most commonly triggered (17).These differences in flagging may be related to the slightly different technologies employed by the analysers as well the specific settings of each analyser in the laboratory as well as possible differences in pathology across the different health tiers. A study assessing morphology flags from laboratories using the same analyser but from different health tiers would be of interest. The absence or under-representation of some morphology flags in each laboratory limited evaluation of these specific flags, however, this study was conducted as a cross-sectional analysis of a random sample population. Every laboratory that elects to validate these rules would need to ensure adequate representation of each morphology flag to complete the validation.

Kim *et* al from Korea evaluated the Advia, Sysmex and Beckman Coulter analysers and found that over all three analysers the platelet count and IG flags accounted for the largest proportion of PBS reviews. The Sysmex analyser had nearly double the cases triggered by IG, ALYM, PCL and large platelets when compared with the other analysers. This mirrors the flags noted in this study in the laboratory using the Sysmex analyser. The Beckman Coulter had greater number of RDW triggers than the other analyser which contrasts the finding of this study where no RDW abnormalities were triggered. This is more likely explained by the patient population in the respective hospitals than analyser differences. The only comment Kim makes regarding the Advia flags is that fewer samples with monocytosis or red cell fragments were triggered (19).

4.4 Sensitivity, Specificity and Efficiency of the ICGH rules.

The sensitivity and specificity of the ICGH rules at the HJH laboratory were ~90% and 60% respectively with efficiency of ~80%. When compared to other recent studies performing similar

evaluations in the developing world in large academic hospitals, the results attained from the tertiary laboratory showed similar results. In this study we show a higher sensitivity and lower specificity than Comar *et al* from Brazil, who had a sensitivity of 77.19% and a specificity of 67.00%(18). This study was also performed in an academic centre (with oncology) however using a much larger representative sample population of 1977 samples. Nonetheless, the efficiency of the ICGH rules was substantially poorer than that seen in the South African tertiary laboratory (70%). The study done in Thailand by Pratumvinit *et al* was performed in the largest hospital in Thailand with a bed capacity of >2000 beds. This hospital is the referral centre for all hospitals in Thailand and would be the equivalent of the central academic hospitals in South Africa. A total of 2114 samples were assessed in the study but unfortunately sensitivity and specificity were not published. The efficiency rates published by this group for the ICGH rules, however, was similar to our tertiary laboratory result (83.63%) (24). Eldanasoury *et al* from Egypt had results similar to the Brazilian group and showed a lower sensitivity (82.13%), higher specificity (78.32%) and similar efficiency (80.37%) to our results. This study was performed in a large university hospital with >3000 beds using 800 samples (17). The reason for the differences in results is likely due to the smaller sample size in this study (divided over three different laboratories) as well as inherent population differences. These differences stress the need for each laboratory to validate these rules and optimise accordingly.

A similar pattern was seen in the regional laboratory, where the sensitivity and specificity of the ICGH rules were ~94% and ~45% respectively, with efficiency of ~75%. When comparing to the other studies using Sysmex analysers, this study had a greater sensitivity and efficiency but lower specificity than Comar *et al*(18) and Kim *et al*(sensitivity and specificity 60% and 77% respectively) (19) but a lower efficiency than Pratumvinit *et al* (74.5% vs 83.6%) (24).

In the district laboratory, the ICGH rules had resulted in a sensitivity of >90% and a specificity of 37.3%. The efficiency was greater than when the laboratory SOP rules were applied to the same

samples. Overall, the efficiency was the least out of all the laboratories assessed (68.6%) and lower than the efficiency rates noted in the literature . This finding appears to reflect a poorer performance of the consensus rules in hospitals that serve less ill patients (district hospital) with a higher proportion of normal/near-normal counts. This finding suggests that the greatest use of ICGH rules is in the central and tertiary laboratories where there are a greater number of abnormal samples.

4.5 False Positives and possible rule optimisation

In the tertiary tier laboratory the FP rate was 15.3%, which is somewhat higher than that reported, by Kim et al in their study assessing three different analysers, where the Advia had the lowest FP rate (11.3%). The HCVAR, LS and WCC <4 x 10^9/L were the flags associated with the highest FP rates.

Of the FP samples which had a LS flag, it was triggered in isolation in 2/3 of samples. This suggests that the LS flag may not be a reliable flag to guide manual review of the smear when seen in isolation.

Based on the FP sample analysis, it was determined that excluding the HCVAR flag would reduce the review rate by 3.4%, while increasing the FN rate by 1.5%. There was only one sample in which the HCVAR flag was triggered and the Hb was <7g/dL. This sample had no abnormal morphology and was, in fact, assessed as FP. Therefore, optimising the flag by adding the rule HB<7g/dL would not aid in reducing the review rate.

The only rule modification that could be entertained was excluding smear review in samples with a WCC from 2-3.99 x 10^9/L, which would reduce the review rate by 3.8% without increasing the FN rate for clinically significant morphology. This finding is particularly pertinent for the South African context considering the lower WCC described in African patients (23), and is further

supported by the recommendations of the Francophone group who suggested that a low WCC is not a good indication for smear review except if a qualitative flag suggests interference with the WCC requiring validation of the WCC result by smear assessment (20). Although excluding the LS flag would reduce the review rate by ~3%, while increasing the FN rate by ~1.5%, the HCVAR and LS flags could not be excluded as they occasionally detected clinically significant morphology when triggered in isolation. Adjustment of the analyser sensitivities of these flags may be of benefit in further reducing the FP rate.

The overall FP rate for the regional tier laboratories was 21.5%, with the most common FP flags being the ALYM, IG, PLC and WCC<4 x 10^9/L flags. These findings are very similar to those of Comar *et al,* who found FP rates of 21.93% and 28.46% using the Sysmex XE-2100D and XT2000i analysers respectively in a much larger cohort (n=1977) (18). The main rules producing FP results in this study included leucocytes<4 x 10^9/L, plts <100 x 10^9/L and unspecified suspect flags.

In our cohort, excluding the ALYM flag would reduce the review rate by 2.5% while increasing the FN rate by 1.5% and with a sensitivity of >70%, excluding the IG flag would reduce the review rate by 5% while increasing the FN rate by an unacceptable 3%. However, exclusion of either of these flags was not considered as both detected clinically significant morphology.

One could consider reducing the Hb threshold from 7 g/dL to 5 g/dL, however, as this was only evaluated in one sample this would require further investigation in a larger cohort. This modification in this cohort would reduce the review rate by 2.5% and increase the FN rate by 0.5%.

Considering the four of the most triggered flags (i.e. ALYM, PLC, IG and WCC<4), one or more were triggered in 91 patients, 65 of which (n=91, 71.4%) were regarded as TP. Excluding all 4 of these flags would be likely to increase the FN rate unacceptably. Therefore, the possibility of

excluding only the PCL and the WCC2-4 x 10^9/L flags was evaluated further, as exclusion of these flags in isolation did not increase the FN rate. Eighteen samples had only the PCL and/or WCC 2-3.99 x 10^9/L flags triggered, 8 of which were TP. The morphology detected in these samples included PCL (in patients with normal platelet counts), large platelets, LS, RF and/or TG. Removing both of these flags would reduce the review rate by 9% while increasing the FN rate by 4%, however the morphology detected in these FN samples was not considered to be of major clinical significance (15).

Caution would need to be exercised in implementing these modifications with respect to the PCL rule, as the detection of platelet clumping in patients with low grade thrombocytopenia (100-149x10^9/l) would be of some clinical value. For this reason, modification of this rule should perhaps only be considered in conjunction with normal/high platelet counts. Adjustment of the analyser sensitivities for the ALYM and IG flags may also be of benefit in further reducing the FP rate.

The district tier laboratory had the highest FP rate (26.4%), with the most common FP rules triggered in isolation being the NRBC and WCC<4 x 10^9/L flags. These flags never detected clinically significant morphology, and disregarding these flags could therefore be considered. The NRBC flag was the least reliable in this sample population as ~70% of the time this flag was triggered in isolation and resulted in a FP flagging suggesting that this flag triggered in isolation may not be a reliable indicator of pathology. NRBCs were triggered in 65 cases. Thirty of these were TP with 6 (n=30; 20%) being triggered in isolation. All of the latter had normal/near-normal counts and the morphology detected included ALYMs in three patients, large platelets in two patients and TG in one patient. Optimisation of this flag including changing the WCC vote-out limit or SL cut-off (as discussed in Table 1.1) could reduce the manual review rate by >20%.

Together, the WCC<4 flag and the NRBC flag were the only flags triggered in 34 cases. Excluding both of these flags would reduce the review rate by 24%while increasing the FN rate by 7.9%. None of the morphology missed in the FN samples was clinically critical.

Unfortunately, as no samples had a WCC <2 x 10^9/L in this sample population, the value of smear review in samples with WCCs in this range has not been assessed. It may thus be prudent to adjust the rule to exclude smear review only in samples with a WCC in the range of 2-3.99^9/L as was proposed in the regional and tertiary laboratories. In comparison with the other Beckman Coulter results published, the overall FP rate of the district laboratory was much higher (26.4% versus 13.7% (Kim *et al*(19)) and 10% (Eldanasoury*et al* (17)). Although these studies were also performed in the developing world, they assessed patients in large academic hospitals with oncology centres and used larger samples (800 and 1485 respectively). The finding of a higher FP rate is likely related to the population studied and health tier differences between this study and the other studies published considering the poorer performance of the consensus rules in hospitals that serve less ill patients (district hospital) and a higher proportion of normal/near-normal counts.

4.6 False Negatives

4.6.1 Tertiary tier laboratory (HJH)

The FN rate in the tertiary tier laboratory was reduced from 11% to 5.8%. The accepted FN rate in the literature stands at 5% (16) and minor adjustments through optimisation of the ICGH rules may improve this rate further.

Laboratory SOP

Samples which were missed included 14 samples with NRBCs (n=260, 5.4%), eight samples with LS (n=260, 3.1%), five samples with ALYM (n=260, 1.9%), four samples with red cell fragments (n=260, 1.5%), four samples with sickle cells (n=260, 1.5%), and two samples with blasts (n=260, 0.8%). Of the 31 samples assessed as FN by laboratory SOP, six (19.4%) had other

parameters which would have triggered a comment suggesting a diff count on the authorised FBC report (as per laboratory SOP). These smears would not have been manually assessed at the time of presentation.

ICGH rules

The most common morphology missed using the ICGH rules were ALYMs in four samples (n=260, 1.5%). The ALYMs in all of these samples were noted within a heterogeneous population of lymphocytes where the majority had, in fact, normal lymphocyte morphology although the numbers of ALYMs were >5 and they did qualify as positive morphology according to the ICGH guidelines. The lymphocytes in all of these samples were favoured to be reactive in nature and further workup would not have been suggested for any of these. Of some concern, review was also omitted in a single sample with blasts. The latter sample had completely normal counts with no flags triggered. On PBS review the blasts accounted for ~3% of white cells and were noted in the context of a left shift. Also of note was the presence of 9 NRBCs/100 WBC. Neither NRBCs nor blasts were flagged by the analyser in this sample. Considering the pathology missed, an overall FN rate of 5.8% is unacceptable.

In contrast, the Advia results in Kim *et al*'*s* study revealed a much higher FN rate of 14.3%. RBC morphology and PLT morphology accounted for the clear majority of FN samples. In the study by David and Schapkaitz PCL and red cell fragments also had the highest false negative rate of the cohort studied at 30.0% and 16.4% respectively (22).

4.6.2 Regional tier laboratories (Tambo Memorial/Sebokeng)

In the regional tier laboratory, the FN rate was reduced from 5.0% to an ideal 3.5%, however, this was done at the expense of an increased FP rate (17.5% to 21.5%).

Laboratory SOP

Samples which were missed included two with ALYM (n=200, 1.0%) and three samples with LS (n=200, 1.5%).Of the two ALYM samples, one had a range of heterogeneous lymphocytes which were favoured to be reactive in nature, however, the other sample revealed a greater majority of ALYMs some of which were somewhat primitive, and ongoing monitoring of the counts and the PBS would have been suggested. The samples with LS included two samples with a mild, likely clinically insignificant LS while one sample revealed numerous pseudo-pelgerised neutrophils along with left shift and toxic granulation. Although it was considered that this finding may be storage-related or artefactual, a repeat sample with ongoing monitoring would have been suggested for confirmation. Of the five samples discussed only one had morphology flags triggered which would have prompted the reviewer to do PBS review had morphology flags been included in the SOP.

ICGH rules

The morphology missed in the FN samples was marginally better using the ICGH rules. Samples which were missed included two samples with LS (n=200, 1%) and one sample with ALYM (n=200, 0.5%). Interestingly, these samples were some of the same ones missed using laboratory SOP and the ICGH rules did not miss any samples that were correctly picked up using the laboratory SOP. The ALYM sample missed by the ICGH rules was the sample where ongoing monitoring would have been advised and the LS samples missed included one clinically less significant sample and the one sample with pseudo-pelgerised neutrophils requiring either repeat sampling or ongoing monitoring.

The FN results reported in studies using the same analyser showed a range from 2.2% (24) to 10.7% (25), and the FN rate reported in this study therefore compares very favourably on implementation of the ICGH rules. Keeping this in mind, the optimisation strategy employed in

this health tier would be focused on reduction of the number of FP samples in an effort to reduce the review rate.

4.6.3 District tier laboratory (Dr Yusuf Dadoo)

The biggest reduction in FN rates was seen in the district tier laboratory with a reduction from 32% to 5%, but this was accompanied by the largest increase in FP rates (6% to 26%).

Laboratory SOP

The laboratory SOP rules had an unacceptably high FN rate of 32.1%, the highest of all three health tiers. The morphology missed using the laboratory SOP included red cell (RF in 14 samples (n=140, 10.0%)), white cell (ALYM in 14 samples (n=140, 10.0%) and LS in 10 samples (n=140, 7.1%)) and platelet derangements (PCL in 8 samples (n=140, 5.7%)). The majority of ALYM samples revealed a heterogeneous range of lymphocytes, however, there were 2 samples where the lymphocytes looked somewhat primitive and ongoing monitoring would have been advised. Of the samples missed with LS, the majority just made the cut-off of LS as defined in the ICGH rules having just one myelocyte. There were 2 samples with more significant LS which may have warranted ongoing monitoring. The platelet clumping samples missed using the laboratory SOP had platelet counts >100 x 10^9/L and the PCL was unlikely to have been clinically significant in the majority of samples. One sample had a borderline platelet count of 149 x 10^9/L which would likely have increased to the normal range for age if the platelet clumping had been detected.

ICGH rules

Similar morphology was missed using the ICGH rules but in fewer samples with the exception of ALYMs and PCL which were only missed using the laboratory SOP. Those missed included four samples with LS (n=140, 2.9%), two of which had more significant LS (moderate numbers of left shifted forms) and RF in three samples (n=140, 2.1%). Much like the regional laboratory, there

were no FN samples triggered using the ICGH rules that were not also FN using the laboratory SOP.

The Beckman Coulter in Kim *et al*'s study revealed a FN rate with the same value as for the Advia in their series (14.3%) which was a lot higher than the current study. Eldanasoury *et al* (also using a Beckman Coulter) reported a FN rate of 9.25%. Both of these studies were conducted in a much larger academic hospital with patients that were likely to have a greater degree of pathology with fewer normal samples than those patients at Dr Yusuf Dadoo hospital, which may account for the lower FN rate.

4.7 Clinically significant morphology

Examples of the importance of deciding on clinically significant morphology were found in each of the three health tiers assessed. In the tertiary laboratory the HCVAR flag was triggered in isolation in 4 instances. Two of these had more clinically significant findings (as defined by Bain) (15) (one a leukoerythroblastic reaction (worrying for possible bone marrow infiltration) and the other spherocytes (features of haemolysis)), while the other two showed less critical findings (ALYMs within a population of heterogeneous lymphocytes in one and target cells with NRBCs (4/100 WBC) in the other. Neither of the latter two samples were considered to be of major clinical significance although they did qualify as positive smear findings using the ICGH criteria.

In the regional laboratory the IG flag was triggered in isolation in six TP samples. One of these samples had clinically significant morphology of blasts while the others revealed reactive/infective changes which were not deemed to be clinically critical.

Another example from the regional laboratories which introduced the concept of the "non-critical FN rate" was noted when looking at the PCL and WCC from 2-3.99 x 10^9/L. Eighteen samples had only the PCL and/or WCC2-3.99 x 10^9/L flags triggered, eight of which were TP. No critical pathology was missed in any of these instances (PCL (in patients with normal platelet counts),

large platelets, LS, RF and/or TG). It was found that removing both of these flags would reduce the review rate by 9% while increasing the non-critical FN rate by 4%.

In the district laboratory the NRBC flag was the least reliable in this sample population as ~70% of the time this flag was triggered in isolation and resulted in a FP flagging. Thirty TP cases were noted with six being triggered in isolation. All of the latter had normal/near-normal counts and the morphology detected was not clinically critical (ALYMs in three patients, large platelets in two patients and TG in one patient).

4.8 Laboratory managers' feedback

Two laboratories (HJH and Sebokeng) report not having enough staff for the Diff bench to cover night shift with subsequent time off or if technologists need a leave of absence. This puts the bench and the remainder of the staff under considerable strain. HJH is the only laboratory with monospecialist technologists in haematology but the other laboratories do have clinical pathology technologists trained and competent in signing out peripheral smear review comments and Diff counts.

The concerns raised by the laboratory managers in reply to the initial questionnaire highlight the importance of introducing validated smear review rules which will reduce the manual review rate while maintaining an adequate FN rate. Of particular note, the laboratory managers from the tertiary and regional laboratories both report a need for possible optimisation of the smear review rules.

4.9 Limitations

This study has a number of limitations which could be addressed by the individual laboratory choosing to perform a similar validation in the future. A limitation which affected all health tiers but had the greatest impact on the district laboratory was the representation of samples in the randomly selected sample population. In an effort to select a randomly representative sample

reflective of the laboratory's patient profile, certain morphology flags were not triggered in enough samples for assessment. In the tertiary and regional laboratories this was the fragment flag and in the district laboratory this was LS, IG, Blasts and ALYM flags. Lack of representation in these laboratories then did not allow for validation of these flags individually and were not triggered in a large enough proportion of the samples for statistically significant optimisation to follow.

For this reason, every laboratory that implements these rules in order to reduce manual smear review rates must ensure they use a sample which is not only representative of the samples seen in the laboratory but also enough samples for each parameter and morphology flag to have acceptable representation for optimisation.

The tertiary tier laboratory (HJH) had a higher Diff count proportion than what is routinely seen in the laboratory. In this study 66% of samples were manually reviewed owing to differential count request by the doctor whereas the statistics reported by the laboratory manager over the same time period reflect a lower number of differential counts (~10%) of total FBC samples. This sample bias did skew the review rate in this study and may not have been a true reflection of standard practice at the laboratory.

4.10 Future recommendations

When starting this study the main goal was to assess the possibility of reducing the manual smear review rate in a laboratory representative of each health tier using the ICGH consensus rules. From the data analysed it is evident that implementation of the rules with certain modifications and optimisation could aid to reduce the review rate in tertiary tier laboratories where the smear workload is at its greatest. As for the regional and district tier laboratories, adoption of a validated rule set which makes use of parameter and morphology flags is imperative, not only to prevent every smear from being manually assessed but also to ensure an acceptable FN rate and avoid

missing clinically significant morphology. Performance of the ICGH rules was poorer in the smaller laboratories which reported a greater number of normal/near-normal blood results. In the South African context, Joubert *et al* recorded results which were noted to be very similar to those attained using the regional (Sysmex) laboratory SOP with only a slightly better specificity (25). As the study by Joubert *et al* was not an analysis of the ICGH rules but rather of the Sysmex analyser and performed in two regional tier laboratories in South Africa, this is an encouraging similarity which reveals reproducibility of results.

Assessment of the data allowed for tier specific as well as a general recommendation to be made for each tier:

4.10.1 Tertiary laboratory (HJH – Advia 2120)

1) The Diff count in this sample population accounted for 66% of the manual smear reviews. While this percentage is suspected to be greater than that seen in reality, request for a Diff count alone should not be in the criteria for smear review.

2) Reduction of the WCC from 4 x 10^9/L to 2 x 10^9/L as a white cell parameter rule for smear review is recommended as this modification would reduce the review rate by 3.8% without missing critical pathology. This is the only FP flag that could be modified as the morphology flags responsible for increasing the FP rate did sometimes pick up critical pathology. A consideration could be made to modify the HCVAR rule to prompt review only in those samples with a Hb<7 g/dL, however, the full effect of this rule combination could not be assessed as it was only noted in one sample. Adjustment of the analyser sensitivities of the HCVAR and LS flags may be of benefit in further reducing the FP rate.

4.10.2 Regional laboratories (Tambo Memorial and Sebokeng Hospitals – Sysmex XT2000i)

1) Modification of the WCC threshold to that of <2 x 10^9/L as this would reduce the review rate without an impact on the clinically significant FN rate.

2) Modification of the PCL rule is also recommended, however, this rule should only be excluded in the presence of normal/increased platelet counts.

3) Exclusion of the IG and ALYM flags could not be endorsed owing to the morphology picked up in the samples where these flags were triggered, however, an argument could be made for adjusting the analyser sensitivities to these flags. This went beyond the scope of this study.

4.10.3 District laboratory (Dr Yusuf Dadoo Hospital –Beckman Coulter Act5diff)

1) Exclusion of the NRBC flag would reduce the manual review rate by >20% without missing clinically significant pathology. Should this be deemed too extreme for some laboratories, adjustment of the analyser's NRBC flag to detect NRBC>5 would also likely still reduce the manual review rate.

2) PCL flag should be excluded in samples with normal/high platelet count.

3) The WCC could be modified to WCC<2 x 10^9/L in order to reduce the manual review rate

4.10.4 General recommendation

In this study, I provide examples of where the ICGH rules performed well as well as optimisation strategies for each laboratory. There is one recommendation of the ICGH consensus which is pertinent to all tiers and that is the validation and optimisation of the positive smear criteria as stipulated by the ICGH. These rules would need to be validated for the South African setting,

considering the concept of clinically significant morphology (15) and the unique pathology encountered in our population. A similar suggestion was made by Comar *et al*(18) in which they mention these criteria are international standards and one would need to clarify if these criteria require optimisation before validation of the consensus rules could take place.

5. Conclusion:

The capabilities of the modern automated analysers are well known and valued but despite the massive improvements in technology there remains a need for manual smear review, particularly in samples with abnormal results. In South Africa, the higher proportion of abnormal results reflects the increased burden of disease brought about by the high HIV prevalence and associated TB epidemic. Employing a version of the ICGH rules for smear review that have been optimised for these very conditions that takes into account the unique pathology encountered in our hospitals, could aid in reducing the review of unnecessary smears while maintaining an adequate level of pathology detection. Each laboratory wishing to employ such rules will need to validate them for their specific analyser and sample population.

References

1. Verso ML. FIRST AUSTRALIAN MEDICAL CONGRESS : section of the history of medicine. Med Hist. 1962;6(4):396-7.

2. Lehner J Greve Band Cassens U. Automation in Hematology. Transfusion Medicine and Hemotherapy. 2007(34):328-39.

3. Oliver G. The CROONIAN LECTURES: A CONTRIBUTION to the STUDY of the BLOOD and the CIRCULATION: Delivered before the Royal College of Physicians of London. Br Med J. 1896;1(1852):1548-50.

4. Green R, Wachsmann-Hogiu S. Development, history, and future of automated cell counters. Clinics in laboratory medicine. 2015;35(1):1-10.

5. Moldavan A. Photo-Electric Technique for the Counting of Microscopical Cells. Science. 1934;80(2069):188-9.

6. Coulter WH, inventor. Means for counting particles suspended in a fluid. USA patent 3781112A. 1953.

7. Coulter WH. High speed automatic blood cell counter and cell size analyser. National Electronics conference; 1956; Chicago.

8. Hansen WP, Hoffman RA. Method and apparatus for automated identification and enumeration of specified blood cell subclasses. Google Patents; 1981.

9. Dittrich W, Gohde W Inventor; Flow-through chamber for photometers tp measure and count particles in a dispersion medium.1968.

10. Ward PC. The CBC at the turn of the millennium: an overview. Clin Chem. 2000;46(8 Pt 2):1215-20.

11. Groner W, George WV, inventor; Technicon Instruments Corporation (Tarrytown, NY), assignee. METHOD AND APPARATUS FOR ANALYSIS OF LEUKOCYTES USING LIGHT SCATTERED BY EACH LEUKOCYTE AT ABSORBING AND NON-ABSORBING WAVELENGTH. United States1973.

12. Kurec A. Flow cytometry: principles and practices. MLO Med Lab Obs. 2014;46(5):28, 30-1.

13. Rappaport ES, Helbert B, Beissner RS, Trowbridge A. Automated hematology: where we stand. South Med J. 1988;81(3):365-70.

14. Zandecki M, Genevieve F, Gerard J, Godon A. Spurious counts and spurious results on haematology analysers: a review. Part II: white blood cells, red blood cells, haemoglobin, red cell indices and reticulocytes. Int J Lab Hematol. 2007;29(1):21-41.

15. Bain BJ. Diagnosis from the blood smear. N Engl J Med. 2005;353(5):498-507.

16. Barnes PW, McFadden SL, Machin SJ, Simson E. The international consensus group for hematology review: suggested criteria for action following automated CBC and WBC differential analysis. Laboratory hematology : official publication of the International Society for Laboratory Hematology. 2005;11(2):83-90.

17. Eldanasoury AS Boshnak NH, Abd El Monem RE. Validation of criteria for smear reviewfollowing automated blood cell analysis in Ain Shams University Laboratory. International Journal of Science and Research. 2016;5(3):484-93.

18. Comar SR, Malvezzi M, Pasquini R. Are the review criteria for automated complete blood counts of the International Society of Laboratory Hematology suitable for all hematology laboratories? Rev Bras Hematol Hemoter. 2014;36(3):219-25.

19. Kim SJ, Kim Y, Shin S, Song J, Choi JR. Comparison study of the rates of manual peripheral blood smear review from 3 automated hematology analyzers, Unicel DxH 800, ADVIA 2120i, and XE 2100, using international consensus group guidelines. Arch Pathol Lab Med. 2012;136(11):1408-13.

20. Genevieve F, Galoisy AC, Mercier-Bataille D, Wagner-Ballon O, Trimoreau F, Fenneteau O, et al. Smear microscopy revision: propositions by the GFHC. feuillets de Biologie. 2014;56(317):1-9.

21. Department of Health. National Health Insurance for South Africa: Towards universal health coverage. Version 40.December 2015. Available from https://www.bhfglobal.com/wp-content/uploads/2015/03/national_health_insurance_white_paper_10Dec2015.pdf

22. David M, Schapkaitz E. Evaluation of morphology flags on the Advia 2120 haematology analyser at a Large Academic Hospital. Poster presentation: African Society of Laboratory Medicine; Cape Town, South Africa2014.

23. Haddy TB, Rana SR, Castro O. Benign ethnic neutropenia: what is a normal absolute neutrophil count? J Lab Clin Med. 1999;133(1):15-22.

24. Pratumvinit B, Wongkrajang P, Reesukumal K, Klinbua C, Niamjoy P. Validation and optimization of criteria for manual smear review following automated blood cell analysis in a large university hospital. Arch Pathol Lab Med. 2013;137(3):408-14.

25. Joubert J, Weyers, R. Raubenheimer, J. Reducing unnecessary blood smear examinations: can sysmex blood cell analysers help? Medical Technology SA. 2014;28(1):6-12.

A. APPENDIX A: Analyser principles and specifications

Table A.1 Analyser specifications of three analysers assessed in this study

Specifications	Advia 2120	Sysmex XT2000i	Beckman Coulter Act5diff
Tier	Tertiary	Regional	District
Throughput	FBC: 120 Samples/hr FBC/diff: 120 Samples/hr FBC/diff/retic: 74 Samples/hr	80samples/hr (max.)	60 samples/hr
Database storage	10 000 records	10 000 records	
Parameters	**FBC Results:** WBC, RBC, HGB, HCT, MCV, MCH, MCHC, CHCM, RDW, HDW, CH, CHDW, PLT **Differential Results** (absolute and %): NEUT, LYMPH, MONO, EOS, BASO, LUC (Large Unstained Cells) **Platelet Results:** PLT, MPV, PDW, PCT **Flags:** WBC: Left Shift, Atypical Lymph, Blasts, Immature Granulocytes, MPO Deficiency ANISO, MICRO, MACRO, HC VAR, HYPO, HYPER, RBC Fragments, RBC Ghosts, NRBC, Platelet Clumps, Large Platelet	**FBC Results:** WBC,RBC,HGB,HCT,MCV, MCH,MCHC, PLT(Impedance and Fluorescence) **Differential Results** (absolute and %): NEUT, LYMPH, MONO, EO,BASO, RDW-SD, RDW-CV,MPV **Platelet Results:** PLT **Flags:** Immature granulocytes, Blasts, Atypical lympho, Left shift, NRBC, RBC lyse resistance, RBC agglutination, Turbidity, Fragments, Iron deficiency, Platelet clumping	**FBC Results:** WBC,HGB, HCT, MCV, MCH, MCHC, RDW, PLT, MPV, PCT,PDW **Differential Results** (absolute and %): NEUT, LYMPH, MONO, EO, BA. **Platelet Results:** PLT **Flags:** Atypical lymphocytes, large immature cells, schistocytes, left shift, blasts, cold agglutinins, platelet aggregates, NRBCs

B. APPENDIX B: Consensus Rules for Review of Automated Full Blood count and White cell Differential

Table B.1Consensus rules for review of automated FBC and Diff (16)

Full blood count parameters

Rule #	Parameter	Primary	Secondary	Tertiary	4th	Action 1	Action 2	Action 3
1	Neonates	First sample				Slide review		
2	WBC, RBC, HGB, PLT, Retics	Exceeds Linearity				Dilute sample & rerun		
3	WBC, PLT	Lower than lab verified instrument linearity				Follow lab SOP		
4	WBC, RBC, HGB, PLT	Vote out				Check sample for clot	Rerun sample	If persists, perform alternate counting
5	WBC	<4.0 or >30.0	AND first time			Slide review		
6	WBC	<4.0 or >30.0	AND Delta failed	AND within 3 days		Slide review		
7	PLT	<100 or >1000	AND first time			Slide review		
8	PLT	Any value	AND Delta failed			Slide review		
9	HGB	<7g/dl or >2g/dl above the upper range for age & sex	AND first time			Slide review	Verify sample integrity	
10	MCV	<75fl or >105 fl	AND first time	Specimen <24 hours		Slide review		
11	MCV	>105 fl	AND adult			Slide review for macrocytic associated changes	Request fresh sample if no macrocytic changes	Report with comment if no fresh sample
12	MCV	Any value	AND delta fail			Verify sample integrity/identity		
13	MCHC	>2 units above the upper limit of reference range				Check for lipaemia/haemolysis/RBC agglutination/spherocytosis		
14	MCHC	<30	AND normal/high MCV			MCHC can be affected by technique of venepunture.		
15	RDW	>22	AND first time			Slide review		

Differential Count parameters

Rule #	Parameter	Primary	Secondary	Tertiary	4th	Action 1	Action 2	Action 3
17	Neut #	<1.0 or >20.0	AND first time			Slide review		
18	Lymph #	>5.0 (adult) or >7.0 (<12 yrs. old)	AND first time			Slide review		
19	Mono #	>1.5 (adult) or >3.0 (<12 yrs. old)	AND first time			Slide review		
20	Eos #	>2.0	AND first time			Slide review		
21	Baso #	>0.5	AND first time			Slide review		
22	NRBC #	Any value	AND first time			Slide review		

Reticulocytes

23	**Retic absolute #**	**>0.100**	AND first time			**Slide review**		

Suspect flags

24	Suspect flag (except ImmG/Band)	Flag+	AND first time	Adult		Slide review		
25	Suspect flag	Flag+	AND first time	Child		Slide review		
26	WBC unreliability flag	Flag+	Any			Check sample integrity and rerun sample		
27	RBC fragment	Flag+	Any			Slide review		
28	Dimorphic RBC	Flag+	AND first time			Slide review		
29	Lyse resistant RBC	Flag+	Any			Review WBC histogram		
30	PLT clumping	Any value				Check sample for clots		
31	PLT flags	PLT & MPV flags without PLT clumps				Slide review		
32	Immature granulocyte	Flag+	AND first time			Slide review		
33	Immature granulocytes	Flag+	AND previous confirmed result	+ delta check fail for WBC		Slide review		
34	Left shift	Flag+				Follow lab SOP		
35	Atypical/variant lymphs	Flag+	AND first time			Slide review		
36	Atypical/variant lymphs	Flag+	AND previous confirmed result	+ delta check fail for WBC		Slide review		
37	Blast flag	Flag+	AND first time			Slide review		
38	Blast flag	Flag+	AND previous confirmed result	Delta check pass or – delta for WBC		Follow lab SOP		

Rule #	Parameter	Primary	Secondary	Tertiary	4th	Action 1	Action 2	Action 3
40	NRBC flag	Flag+				Slide review		
41	Retics	Abnormal pattern				Look at instrument output		

C. APPENDIX C: HREC Ethics Certificate

R14/49 Dr Nikki Bouwer M130724M130724

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M130724

NAME: (Principal Investigator)	Dr Nikki Bouwer
DEPARTMENT:	Haematology and Molecular Medicine University of Witwatersrand
PROJECT TITLE:	Validation of Haematology Slide Review Rules at Various Tiers of Diagnostic Laboratories in South Africa
DATE CONSIDERED:	26/07/2013
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Mahlangu/Dr Schpkaitz/Dr Vaughan
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 16/08/2013

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature

1/08/2013
M130724Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

D. APPENDIX D: Public Hospital Tiers(21)

Table D.1 Public hospital tiers

Tier	Service Offered
District hospital	Provide generalist medical services including Obstetrics and Gynaecology, Paediatrics, General surgery and Family Medicine.
Regional hospital	Specialist support in the eight core specialities including Obstetrics and Gynaecology, Paediatrics, General surgery, Emergency Medicine, Orthopaedics, Diagnostic Radiology and Anaesthetics. These hospitals offer supervised medical training and have capability and resources for research.
Tertiary hospital	Strong core of specialists which may be available at regional level but with the addition of other specialist and sub-specialist services.
Central hospital	Highly specialised services that require unique skilled personnel.
Specialised hospital	Specialised psychiatric services.

E. APPENDIX E: Questionnaire sent to laboratory managers

Good day,

I am working in the Department of Molecular Medicine and Haematology at Charlotte Maxeke Johannesburg Academic Hospital. I am collating data from the regional laboratories and would really appreciate your assistance in this matter:

1) Could you send me your volumes for the months January to March for FBCs requested as well as those requested as FBC and differential count?

2) Would you mind answering these two questions?

a. Do you have enough staff on your haematology bench to cover the work? Do you have specific technologists allocated to morphology (i.e. are there morphologists employed in your lab?)

b. Do you receive complaints from clients (i.e. DOH doctors) about TATs?

F. APPENDIX F: Data Collection

1. Study number (Hospital + number of sample)

2. Demographic information including:

    a. Age

    b. Gender

    c. Ward/clinic

3. FBC alone versus FBC&Diff

4. Full blood count including:

    a. White cell count

    b. Haemoglobin

    c. MCV

    d. MCHC

    e. RDW

    f. Platelet count

5. ICGH rule triggered (Yes=1, No=0)

    a. WCC<4 x 10^9/L

    b. WCC>30 x 10^9/L

    c. Hb<7 g/dL

    d. Hb>2 g/dL more than upper limit of normal

    e. MCV<75fl

    f. MCV>105fl

    g. RDW>22

    h. Plts<100 x 10^9/L

    i. Plts>1000 x 10^9/L

    j. Neuts<1.0 or >20 x 10^9/L

    k. Lymphs>5.0 x 10^9/L

    l. Monos>1.5 x 10^9L

m. Eos>2.0 x 10^9/L

6. Morphology flags triggered (each flag assessed as yes=1, no=0)

7. Peripheral smear morphology present on smear

8. Slide to be reviewed with current SOP (Y/N)

9. Slide to be reviewed using ICGH rules (Y/N)

10. Positive smear finding as per ICGH guidelines (Y/N)

11. True positive/True negative/False positive/False negative