AOSIS

Assessment of the AQUIOS flow cytometer – An automated sample preparation system for CD4 lymphocyte PanLeucogating enumeration

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Background: Flow cytometry has been the approach of choice for enumerating and documenting CD4-cell decline in HIV monitoring. Beckman Coulter has developed a single platform test for CD4+ T-cell lymphocyte count and percentage using PanLeucogating (PLG) technology on the automated AQUIOS flow cytometer (AQUIOS PLG).

Objectives: This study compared the performance of AQUIOS PLG with the Flowcare PLG method and performed a reference interval for comparison with those previously published.

Methods: The study was conducted between November 2014 and March 2015 at 5 different centres located in Canada; Paris, France; Lyon, France; the United States; and South Africa. Two-hundred and forty samples from HIV-positive adult and paediatric patients were used to compare the performances of AQUIOS PLG and Flowcare PLG on a FC500 flow cytometer (Flowcare PLG) in determining CD4+ absolute count and percentage. A reference interval was determined using 155 samples from healthy, non-HIV adults. Workflow was investigated testing 440 samples over 5 days.

Results: Mean absolute and relative count bias between AQUIOS PLG and Flowcare PLG was -41 cells/μL and -7.8%. Upward and downward misclassification at various CD4 thresholds was $\leq$ 2.4% and $\leq$ 11.1%. The 95% reference interval (2.5th – 97.5th) for the CD4+ count was 453–1534 cells/μL and the percentage was 30.5% – 63.4%. The workflow showed an average number of HIV samples tested as 17.5 per hour or 122.5 per 8-hour shift for one technician, including passing quality controls.

Conclusion: The AQUIOS PLG merges desirable aspects from conventional flow cytometer systems (high throughput, precision and accuracy, external quality assessment compatibility) with low technical operating skill requirements for automated, single platform systems.

Keywords: HIV; cluster of differentiation 4, CD4 Enumeration; PanLeucogating.

Introduction

Flow cytometry has been the system of choice for CD4 lymphocyte enumeration and documentation of the decline of CD4 T-cells associated with immunosuppression and lowered counts in HIV-positive patients.[1,2,3] Many diverse CD4 systems that offer solutions to improve and ensure the quality of testing and improve access to testing technologies have been described over the last 25 years.[4,5]

In resource-limited settings, there are many instances where laboratory infrastructure is a limiting factor. However, flow cytometric systems and simpler technologies (such as point-of-care technologies), when used in a tiered laboratory approach, can offer a solution.[6,7,8,9,10] In such an approach, primary centres offer simplified testing and refer testing for flow cytometry analysis to secondary or tertiary centres.[11,12,13] Despite the relative technical complexity, flow cytometry systems, particularly those that require less technical expertise, have been implemented with

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success in some national programmes.[11,12] The suitability of proposed instrumentation must be assessed in the context of the destination laboratory. Concerns such as the level of technical skill required for operation (ease of use, training, and automation), daily sample load and turn-around time requirements, external quality assessment programme compatibility and quality control reagent availability, supplier availability and support, transit requirements, infrastructure, and cost per test should be considered.[11,14]

The PanLeucogating (PLG) CD4 counting method[12,15,16] incorporates a simple gating strategy with only CD45 and CD4 to enumerate CD4 lymphocytes. Quality assessment programmes reported improved performance of PLG CD4 counting and revealed better quality in both the intra- and inter-laboratory reported percent coefficient of variation outcomes.[12,17] Decreased costs of the simplified system also played an important role in addressing some of the aforementioned concerns.[16,18] This method was adopted as the predicate method by the South African National Health Laboratory Service (NHLS) in 2004. The NHLS programme had grown to 35 laboratories by 2007,[12] reaching 60 networked CD4 laboratories by 2014[11] in a tiered system utilising either Beckman Coulter FC500 (Beckman Coulter, Inc., Miami, Florida, United States) or XL (Beckman Coulter, Inc., Miami, Florida, United States) instruments according to service workload requirements.[11]

A single platform volumetric flow cytometer (AQUIOS, Beckman Coulter, Inc., Miami, Florida, United States) was recently developed that utilises a conventional CD4 gating method based on CD45 and CD3 with both CD4 and CD8 for CD4-positive and CD8-positive T-cell lymphocyte counts and percentages. This system was updated in 2013-2014 using bead-based counting for use with the current South African laboratory network PLG predicate[13,14]. The AQUIOS system is fully automated from sample preparation to flow cytometry analysis. It allows for operator independent loading and testing for multiple samples. It has pre-configured panels or protocols that are not modifiable by the user, enabling standardised testing. In line with the tiered model adopted by the National Health Laboratory Service, PLG testing on the AQUIOS system (AQUIOS PLG) was proposed as the system to replace aging and redundant FC500 and XL flow cytometers operational within the South African network, as well as extend its use into small laboratories that offered basic clinical pathology but no CD4 services.[19]

The objective of this study was to compare the performance of AQUIOS PLG with traditional PLG CD4 methods generated on the FC500 instrument at a local South African site, as well as established CD4 reference centre sites in high-income countries. Additionally, normal samples were collected and these data were used to calculate a reference interval to establish whether normal counts generated by AQUIOS PLG matched other published reference intervals.[20,21,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41,42]

Methods

Ethical considerations

All sites had ethics committee or internal review board approval for the collection of samples or use of leftover samples and use of minimal demographic data of age and gender or a waiver was in place for use of these samples. Use of leftover samples for research purposes was agreed to at the time of routine laboratory blood draw. The following ethical clearances were in place: South Africa (identification number: M121020); France (Lyon; identification number: AC-2013-1808); Canada (identification number: 09763E); and the United States (identification number: 10259-05). In France (Paris), consent was given at the time of routine blood draw for the use of leftover samples for research under a waiver according to current French legislation (Loi Jardé, n°2012-300). Patients providing samples for the reference interval study signed a consent form.

Specimens

Method comparison

Patient samples were obtained from incoming routine laboratory specimens for CD4 testing between November 2014 and March 2015 from four different centres located in Canada; Paris, France; Lyon, France and South Africa. A total of 270 samples from HIV-positive adult and paediatric patients were tested, ranging in age from 2 months to 77 years. Thirty samples (22 adults, 8 children) were excluded for the following reasons: CD4 < 20 cells/μL (the instrument's lower limit of quantitation) (18 samples), operator error with manual FlowCount addition (FC500) (4 samples), short blood draw (2 samples), reliability quality control failure (FC500) (2 samples), clot (1 sample), clog (1 sample), insufficient lymphocytes (1 sample) and duplicate patient (1 sample). Testing was performed in duplicate, with replicate 1 used for analysis, except for two samples, for which replicate 2 was used due to system error for replicate 1 (1 high count rate and 1 clog). Two hundred and forty samples were included in the final analysis with specimen ages at time of testing ranging from 20 to 71 hours from collection.

Reference interval in healthy adults

Participants were enrolled between December 2014 and February 2015 from three centres located in Canada; Paris, France and the United States. Samples were obtained from: 1) healthy, non-HIV patient donors who were either hospitalised patients or outpatients with no haematological disease upon final diagnosis (Canada); 2) leftover samples from healthy, non-HIV volunteers donating blood to the Etablissement Francais du Sang (Paris, France); and 3) self-reported healthy, non-HIV participants enrolled through the internal donor programme at Beckman Coulter (Miami, Florida, United States). Participants with CD4 < 300 cells/μL were excluded so as not to include participants with potential idiopathic CD4+ lymphopenia.[20,21] A total of 173 participant samples from healthy adults aged

18–65 years, with normal complete blood counts and differential were tested. Eighteen samples were excluded for the following reasons: haematologic diagnosis (10 samples), lymphopenia (3 samples), participants < 18 years of age (2 samples), lymphocytosis (1 sample), time of collection missing (1 sample) and CD4 < 300 cells/µL (1 sample). One hundred and fifty-five samples were included in the final analysis, all tested within 24 hours of collection.

Workflow

Samples were obtained from incoming routine laboratory samples for CD4 testing at one centre in South Africa over five days in February 2015. A total of 440 participant samples were tested over five days.

Laboratory

Prior to each day's testing, stabilised blood products IMMUNO-TROL Cells (normal CD4 count) (Beckman Coulter, Inc., Miami, Florida, United States) and IMMUNO-TROL Low Cells (Beckman Coulter, Inc., Miami, Florida, United States) passed their assay requirements as quality control material. Samples were collected into ethylenediaminetetraacetic acid vacutainers and tested in duplicate on both instruments for method comparison and only on AQUIOS for reference interval. The PLG gating strategy, used for both instruments, is detailed in Figure 1. All reagents were supplied by Beckman Coulter, Inc., Hialeah, Florida, United States and Immunotech, Marseille, France.

AQUIOS instrument

CD4+ counts and percentages were determined using AQUIOS PLG, as per manufacturer's instructions for use. Each site had an instrument and all required reagents.

FC-500 instrument

Comparator testing was done using FlowCARE PLG CD4 Reagent on the Beckman Coulter FC-500 MCL Flow Cytometer (Flowcare PLG) per the manufacturer's instructions for use. All required reagents were provided. A TQ-Prep™ Workstation (Beckman Coulter, Inc., Miami, Florida, United States) was used for red cell lysing. Sites either used a PrepPlus™ 2 Workstation (Beckman Coulter, Inc., Miami, Florida, United States) for specimen preparation or manual preparation in place of instrumentation.

Statistical analysis

Clinical and Laboratory Standards Institute guidelines EP09-A2 and EP28-A3[43,44] for method comparison[43] and reference interval determination[44] were followed. Replicate 1 was used for analysis, with replicate 2 used for the resolution of replicate 1 discrepancies. For method comparison, the number and percentage for sex, adult or paediatric specimens, mean age, CD4 count and percentage and CD4 count range were calculated. AQUIOS PLG and Flowcare PLG results were analysed using Deming regression to estimate bias at the clinically relevant CD4-positive levels of 50, 100, 200, 350 and 500 cells/µL. Weighted Deming regression was used for count because the variability (scatter) of the data depended on the range of measurements, while simple Deming regression was used for percentages. The coefficient of determination, R^2 (Pearson correlation squared), was used to measure the overall correlation between the two methods. Bland-Altman analysis[45] was used to calculate the mean and median difference between methods. Mean and median relative bias expressed as percent was also calculated. Mean and median absolute difference and relative difference were calculated by CD4 subgroups ≤ 200, 201–1000, and > 1000 cells/µL and ≤ 350, 351–1000, and > 1000 cells/µL, where 200 cells/µL and 350 cells/µL represent antiretroviral treatment (ART) thresholds and 1000 cells/µL was used to control for variability of paediatric samples. Upward and downward misclassification probabilities at ART thresholds of 100, 200, 350 and 500 cells/µL were determined (the method has been described elsewhere[46]). Upward misclassification represents the percentage of additional patients who would fall above a defined threshold with the new test, whereas downward misclassification represents the percentage of additional patients that would fall below this threshold. Mean percent
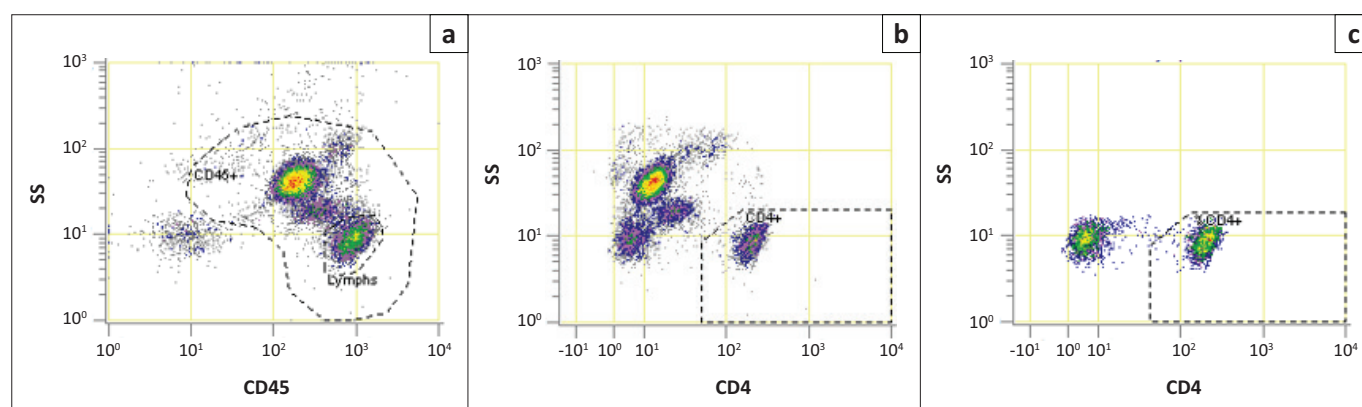
CD4, cluster of differentiation 4; SS, side scatter.

FIGURE 1: CD4 T-cell enumeration by Panleucogating with AQUIOS Flow cytometer, Canada; Paris, France; Lyon, France; and South Africa, November 2014 to March 2015. (a) CD45 versus side scatter plot is used to identify total leukocytes (CD45-positive); (b) All events gated in the CD45-positive region (Pan-Leucogate) are used to plot CD4 versus side scatter to identify CD4-positive lymph cells (CD4-positive Count/µL). (c) Lymphs gate from A is used to plot the CD4 versus side scatter to calculate the CD4 percentage of lymphoid cells (CD4-positive Lymph percent).

similarity with a standard deviation (SD) and coefficient of variance was also calculated (the method has been described elsewhere[47]). For the reference interval, demographic characteristics were calculated (number and percentage for sex and mean ± SD for age). Mean, SD, median, and range for both CD4-positive count and CD4-positive percentage were calculated by sex and for total participants. The 95% (2.5th – 97.5th) reference interval was determined using non-parametric methods. Statistical differences in variables sex, adult or paediatric patient status were determined by *t*-test for the means and non-parametric Mann Whitney U test for

the medians. For workflow analysis, time to first result, average sample results per hour, and sample results per 8-hour shift were calculated. Statistical analysis was performed using Microsoft Excel (Microsoft Corporation, Redmond, Washington, United States) with Analyse-IT.

Results

Method comparison

Two hundred and forty specimens were included in this method comparison study, 92 (38.7%) were obtained from
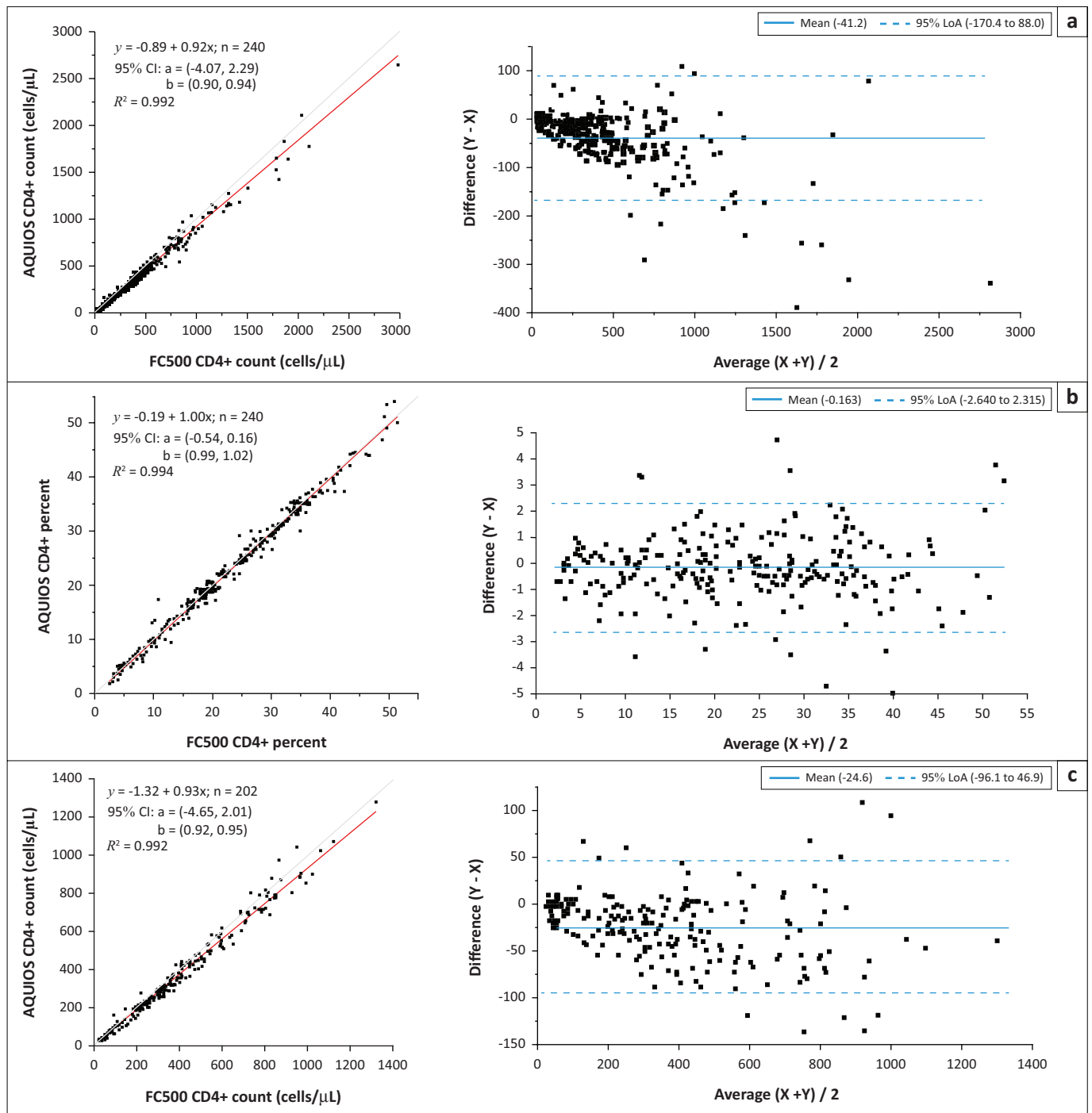
LoA, limits of agreement, CI, confidence interval; CD4, cluster of differentiation 4.

FIGURE 2: Deming regression and Bland-Altman for AQUIOS PanLeucogating versus Flowcare PanLeucogating, Canada; Paris, France; Lyon, France; and South Africa, November 2014 to March 2015. (a) Absolute CD4 count in cells/μL for adults and children, (b) Percentage of CD4 for adults and children, (c) Absolute CD4 count in cells/μL for adults only.

female participants (mean age 35.2 years) and 146 (61.3%) were from male participants (mean age 42.6 years); 202 (84.2%) were adults and 38 (15.8%) were children. Mean CD4 count and percentage were not significantly different by sex (females: 339 cells/μL and 22.65%, males: 359 cells/μL and 23.47%; $p > 0.05$), but were by adult versus paediatric participants (adults: 361 cells/μL and 22.20%, children: 948 cells/μL and 32.28%; $p < 0.001$). The CD4 count range showed a higher minimum–maximum for paediatric specimens (46–2645 cells/μL) than adult specimens (24–1278 cells/μL). CD4 count for all specimens showed an $R^2 = 0.992$ and mean bias of -41 cells/μL between AQUIOS PLG and Flowcare PLG (Figure 2a). Mean relative bias was -7.8%. Of 12 samples with a CD4 count above 1250 cells/μL, 11 were from paediatric participants. For CD4 percentage, R^2 was 0.994 and there was an average bias of -0.16% (Figure 2b). Because the mean CD4 count between adults and children was significantly different, adult specimens were analysed separately, showing an $R^2 = 0.992$ and average bias of -25 cells/μL (Figure 2c). Mean relative bias was -6.8%.

TABLE 1: CD4 count absolute and relative bias between AQUIOS PanLeucogating and Flowcare PanLeucogating overall, by CD4 subgroup and at clinically relevant CD4 levels, Canada; Paris, France; Lyon, France; and South Africa, November 2014 to March 2015.

CD4 count (cells/μL)	N	Absolute difference			Relative difference†	
		Mean		Median (cells/μL)	Mean (%)	Median (%)
		cells/μL	95% CI			
Overall	240	-41	-50 – -33	-27	-7.8	-8.2
Bias by subgroup						
≤ 200	62	-7	-12 – -2	-5	-7.4	-7.8
201–1000	155	-39	-47 – -31	-35	-7.8	-8.1
> 1000	23	-145	-196 – -95	-133	-9.4	-11.4
≤ 350	107	-14	-18 – -10	-12	-7.8	-7.9
351–1000	110	-46	-57 – -35	-45	-7.5	-8.1
> 1000	23	-145	-196 – -95	-133	-9.4	-11.4
Bias by level						
50	-	-5	-8 – -2	-	-9.8	-
100	-	-9	-11 – -6	-	-8.9	-
200	-	-17	-20 – -14	-	-8.5	-
350	-	-29	-34 – -25	-	-8.3	-
500	-	-41	-48 – -34	-	-8.2	-

CD4, cluster of differentiation 4.
†, test – reference / (reference × 100).

TABLE 2: Misclassification percentages at various CD4 count thresholds, Canada; Paris, France; Lyon, France; and South Africa, November 2014 to March 2015.

AQUIOS CD4 count threshold	All		South Africa (%)	Paris, France (%)	Canada (%)	Lyon, France (%)
	%	n/N				
100 cells/μL						
Upward (%)	2.4	1/41	0.0	0.0	0.0	12.5
Downward (%)	1.5	3/199	2.2	0.0	0.0	3.7
200 cells/μL						
Upward (%)	1.6	1/62	0.0	0.0	0.0	8.3
Downward (%)	3.9	7/178	1.2	4.9	3.1	13.0
350 cells/μL						
Upward (%)	0.9	1/106	0.0	0.0	3.4	0.0
Downward (%)	8.2	11/134	8.7	18.2	5.0	0.0
500 cells/μL						
Upward (%)	0.0	0/150	0.0	0.0	0.0	0.0
Downward (%)	11.1	10/90	12.7	0.0	7.7	22.2

CD4, cluster of differentiation 4.

Bias analysis by CD4 subgroups based on ART thresholds of 200 cells per μL and 350 cells per μL showed relatively consistent median relative bias for samples below and above the respective thresholds: -7.8% and -8.1%, and -7.9% and -8.1% (Table 1).

Upward misclassification ranged from 0.0 to 2.4% and downward misclassification ranged from 1.5 to 11.1%, depending on the threshold (Table 2). Mean percent similarity (SD, coefficient of variance) was 96.1% (6.1%, 6.3%) for the absolute CD4 count and 99.5% (3.9%, 4.0%) for the CD4 percentage.

Reference interval in healthy adults

Demographic characteristics for the 155 samples included in the reference interval analysis showed the proportions of female participants were Paris, France, 0.73; Canada, 0.59; United States (0.39). The mean overall ages were 40 years (Canada); 45 years (United States); the mean female-to-male ages were 36 versus 45 years (Canada) and 52 versus 41 years (United States). Specific age data for samples from Paris, France were not available. The mean CD4-positive count ± SD was higher in Canada (1009 ± 239 cells/μL) compared to both the United States (866 ± 221 cells/μL) and Paris, France (802 ± 273 cells/μL). Similar results were seen for the mean CD4-positive count by sex at each site. The 95% reference intervals for both absolute CD4-positive counts and percentages by site were similar and overlapped.

The mean CD4 absolute count and percentage for female and male participants were not different statistically ($p = 0.61$ and 0.48, respectively) (Table 3). The overall mean CD4 positive count ± SD was 888 ± 255 cells/μL and mean CD4-positive percentage ± SD was 46.82 ± 7.86%. The 95% reference interval (2.5th – 97.5th) for CD4-positive count and percentage

TABLE 3: Demographic characteristics and means, medians, and ranges for CD4 and percentages for a healthy adult reference interval, Canada; Paris, France; and United States, December 2014 to February 2015.

Measurement	Sex		p	Overall
	Female	Male		
Demographics				
N	86	69	-	155
Percentage	55.5	44.5	-	-
Age†	43.5	42.2	0.58	42.8
SD	13.0	13.3	-	13.1†
CD4-positive count (cells/μL)				
Mean	879	900	0.61	888
SD	267	241	-	255
Median	874	897	0.61	878
Range (min-max)	352–1573	456–1778	-	352–1778
CD4-positive percentage				
Mean	47.25	46.28	0.45	46.82
SD	7.67	8.12	-	7.86
Median	47.12	46.29	0.48	46.83
Range (min-max)	28.05–69.07	27.90–63.84	-	27.90–69.07

CD4, cluster of differentiation 4; SD, standard deviation.
†, Based on $N = 107$ (age other than between 18 and 60 years not provided by Paris, France site).

TABLE 4: 95% reference interval (2.5th – 97.5th) for apparently healthy adults, Canada; Paris, France; and United States, December 2014 to February 2015.

Overall	CD4 %		CD4 cells/μL	
	Value	90% confidence interval	Value	90% confidence interval
Lower reference interval	30.47	27.90–35.13	453	352–506
Upper reference interval	63.38	60.11–69.07	1534	1329–1778

CD4, cluster of differentiation 4.

TABLE 5: Comparison of overall normal reference intervals (2.5th – 97.5th) for CD4 lymphocytes in HIV-negative adults, Canada; Paris, France; Lyon, France; and South Africa, November 2014 to March 2015.

Region	Study	Technology/platform	N	Age (years)	Sex (% female)	CD4-positive absolute count		CD4-positive percentage 95% Ref Int
						Mean (cells/μL)	95% Ref Int	
United States/Canada/ Europe	This study 2015	AQUIOS/single†	155	18–65	55.5	888	453–1534	30.5–63.4
	AQUIOS Tetra 1 2013[20]	AQUIOS/Single	161	18–65	47.8	904	518–1472	33.6–64.8
	Germany 2005[21]	FACSCalibur/dual†	100	19–84	50.0	870‡	490–1640	30.0–59.0
	Italy 1999[22]	multiple	968	18–70	45.0	940	493–1666	32.0–61.0
Latin America	Mexico City 2013[23]	FACSCount/single	400	20–40	50.0	800	340–1260	NA
Southern Africa	South Africa 2009[24]	EPICS-XL/single†	675	18–55	87.3	1104	548–2045	29.8–58.1
	Botswana 2004[25]	FACSCount/single	437	Adults	32.7	759	366–1318	NA
Eastern Africa	Malawi 2011[26]	FACSCalibur/single	214	Adults	50.5	863	276–1730	NA
	Tanzania 2009[27]	FACSCount/dual	102	> 10	58.8	746	312–1368	NA
	Tanzania 2008[28]	FACSCalibur/single	273	19–48	47.6	802‡	406–1392	27–52
	Tanzania 2003[29]	MultiSET/single	214	17–61	50.0	843	405–1500	27.0–55.0
		SimulSET/dual	214	17–61	50.0	853	403–1604	23.1–54.0
	Kenya 2013[30]	FACSCalibur/single	315	16–60	27.0	920	343–1493	24.0–48.0
	Kenya 2008[31]	FACSCalibur/dual	1293	18–55	34.4	851	421–1550	30.0–55.0
	Eastern Africa 2009[32]	Multiple FACS/dual	2100	18–59	48.6	860‡	457–1628	NA
	Uganda 2011[33]	EPICS-XL/dual†	172	15–70	43.6	938	418–2105	18.8–54.1
	Ethiopia 2014[34]	EPICS-XL/dual	320	18–64	49.7	820	321–1389	NA
	Ethiopia 1999[35]	FACScan/dual	142	15–45	35.2	775	366–1235	NA
Western Africa	Nigeria 2009[36]	Cyflow/single	2570	> 18	47.0	847	365–1571	NA
	Burkina Faso 2007[37]	FACSScan/single	186	18–78	47.8	1082‡	631–1696	30.0–53.0
Indian Subcontinent	Chennai 2009[38]	FACSCount/dual	213	18–56	39.4	926	376–1476	21–59
	India 2003[39]	EPICS-XL/dual	94	18–74	41.5	865	430–1740	30.8–49.6
Asia/Southeast Asia	Singapore 2004[40]	FACSCalibur/single	232	16–65	55.2	838	401–1451	23.0–48.2
	Hong Kong 2013[41]	FC500/single	273	17–59	45.0	760	396–1309	28.1–53.4
	Shanghai 2004[42]	Bryte-HS/dual	614	16–50	38.6	727	415–1189	NA

Ref Int, Reference Interval; NA, not applicable.
†, PanLeucogating used.
‡, Median.

was 453–1534 cells/μL and 30.5% – 63.4% (Table 4). Recent, previously established reference interval results for CD4 absolute count and percentage from healthy, non-HIV individuals from different parts of the world using differing instrument platforms show consistent results (Table 5).

As per protocol analysis that is presented in the AQUIOS PLG test instructions for use, six participant samples from Paris were excluded. These samples had CD4 levels of 300–500 cell/μL. As per protocol, samples with CD4 levels < 500 cells/μL required an in-house medical monitor review of the complete blood count with differential to confirm haematological normal status and qualify for inclusion. The complete blood count with differential results were not provided for samples from the French site, so for the per protocol analysis, those with CD4 < 500 cells/μL were excluded, as it was not possible to exclude the presence of idiopathic CD4-positive T-lymphocytopenia. For this manuscript analysis, these samples with a CD4 ≥ 300 -500 cells/μL were included for the following reasons: 1) the French samples had a confirmed haematologically normal complete blood count with differential performed at the Etablissement Francais du Sang (although results were not available to Beckman), and 2) the Centers for Disease Control does not consider a decreased CD4 level in healthy, non-HIV patients significant unless < 300 cells/μL.[17,18] As expected, the inclusion of these samples decreased the lower reference interval value from 532 cells/μL to 453 cells/μL. The upper reference interval value was not affected and thus remained unchanged.

Workflow

A total of 440 samples were tested over 5 days (Table 6). The average time to the first result was 39.2 minutes. The average number of samples processed per hour was 17.5 or 122.5 for an 8-hour shift, minus 1 hour for start-up, quality control testing and shut-down. Technician hands-on time required one hour, including start-up, quality control testing, sample testing and shut-down.

TABLE 6: High volume workflow results with AQUIOS PanLeucogating, South Africa, February 2015.

Testing day	No. of samples tested	Time to first result (minutes)	Time from start of first sample to result for last sample (hours:minutes)	Samples/h	Samples/8 h shift†
1	105	38	5:50	18.0	126.0
2	86	38	5:09	16.7	116.9
3	72	47	4:26	16.2	113.4
4	90	35	4:39	19.4	135.8
5	87	38	5:07	17.0	119.0
Overall	**440**	**39.2**	**25:11**	**17.5**	**122.5**

h, hour.

†, Includes 1 hour for start-up, quality control, and shut-down.

Discussion

In this study, we compared PLG on the AQUIOS Flow Cytometer versus PLG CD4 counts generated by FC500 instruments. Additionally, CD4 counts generated from normal individuals were used to establish and gain insights into the reference interval of the CD4 counts of patients tested by AQUIOS PLG with respect to other published reference intervals.

The method comparison of AQUIOS PLG to Flowcare PLG showed a mean absolute count bias of -41 cells/μL and a mean relative bias of -7.8% including both adult and paediatric HIV samples. For adults only, a mean absolute count bias of -25 cells/μL with a mean relative bias of -6.8% was observed, similar to outcomes noted in separate evaluations.[48] The slight negative bias in our study appears to be largely platform related and not related to the gating strategy used. Where different gating strategies (AQUIOS Tetra, with primary CD45 bright and CD3 gating to define CD4 lymphocytes and AQUIOS PLG which relies only on CD45 total and CD4/SS to discriminate monocytes) were applied on the same platform, a bias of just 10 cells/μL with an R^2 of 0.996 (unpublished data) was noted. This finding is similar to the slight positive bias previously reported where bead-based counting versus volumetric-based counting comparison was performed.[6] While the mean and median absolute count difference increases with the CD4 level, the relative difference remained stable (around 8% – 11%) across the entire range of samples tested, even at counts above 1000 cells/μL (where clinical importance is less). Paediatric patients are known to have higher CD4 absolute levels and higher variability in these counts than adults,[49] and this was seen in our study as well, where mean and maximum CD4-positive counts were higher for paediatrics than adults.

Misclassification probability measures are used to determine the likelihood that a patient's result for a new test compared to a reference test will be classified above or below a defined threshold used in clinical decision-making.[50,51] These measures provide a more direct interpretation for health policy and management decision making with regard to potential financial and healthcare impacts from implementation of new instrumentation.[51] Misclassification results for CD4 should be interpreted for regions where World Health Organization guidelines[52] for universal testing and treatment are not applied and where varying treatment thresholds still exist. Our results indicate that the introduction of AQUIOS PLG may result in ≤ 2.4% upward and ≤ 11.1% downward misclassification.

The CD4-positive absolute count mean and 95% (2.5th – 97.5th) reference interval among healthy, non-HIV adults from our study were consistent with previously established reference intervals in other studies[20,21,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41,42] on healthy individuals from different parts of the world (Table 5). This consistency of reference intervals established the equivalency of PLG gating methods in the context of reference intervals where typically conventional gating strategies were used. Reference intervals enable meaningful interpretation of a patient's laboratory results. It is recommended that laboratories establish local reference ranges for clinical use[44,53] due to known differences relating to location, race or ethnicity, sex, age, disease burden and drug intake.[21,22,27,28,29,33,36,38,39,42,49,54] In many countries, especially low- and middle-income countries, resources frequently limit local reference range development[24] and there are few published reference interval datasets. Interpretation of reporting is largely based on reference ranges published from high-income, industrialised areas of North America and Europe.[24] Our results, however, indicate that CD4 results across multiple geographies, instruments and platforms are quite consistent, including results for CD4 obtained by PLG on the AQUIOS.

The circumstances and conditions for CD4 testing in laboratories vary in low- and middle-income countries. Challenges faced vary and include a basic lack of infrastructure such as an unstable electricity supply, a lack of cold storage facilities to a paucity of skills.[55] The burden of HIV disease may also frequently dictate high workload volumes in certain countries.[11,12] Manual sample preparation requires multiple pipetting, which in turn increases pipetting errors. However, automated sample preparation lowers percent coefficients of variance through the reduction of human pipetting errors.[12,56] The simplified PLG CD4 method[12,16] is the predicate system of the South African programme, and it combines the reliable bead-based testing technology[8] and a gating strategy.[8,12,16,57] PLG on the AQUIOS platform is regarded as a suitable candidate for this programme to replace older aging equipment currently used[12,48] It is envisaged that its user-independent and on-board sample preparation features could improve testing outcomes in small laboratory sites with fewer staff or less technical flow cytometry expertise. Thus, this enables them to provide local CD4 services even with workload equivalents of up to 100 samples per day and

improve local service delivery turn-around times in more remote parts of South Africa.[11,19]

The workflow showed a medium-high throughput level. Lower throughput (12 samples per hour or 96 samples per shift tested) was seen in a previous workflow study performed in the same laboratory using manual preparation and analysis on a FC-500 flow cytometer, and, 6 hours of hands-on technician time were required.[58] The AQUIOS PLG daily workflow includes available quality control material and the system works with stabilised blood products, making it compatible with external quality assessment programmes. This allows for ongoing monitoring of intra- and inter-laboratory precision which is important for quality management of large-scale country-wide programmes.[11,12] The system tracks quality control results and alerts user and technical support staff of deviations or trending. As a single platform system with on-board sample preparation, no additional laboratory equipment was needed.

Lastly, it is important to briefly discuss the relevance of CD4 counting in the face of recent World Health Organization guidelines[52] which recommend that all patients who are HIV-positive start ART, irrespective of their CD4 counts. For the past 30 years, medical personnel caring for HIV patients have used CD4 counts as prognostic indicators of disease progress[3] or death.[2] Also, the CD4 count is used to determine eligibility for initiating ART, managing and treating opportunistic infections and monitoring the patient's response to ART.[59] It is widely agreed that HIV viral load testing is the optimal assay to monitor the response to ART or determine treatment failure.[60,61] However, in light of the documented worldwide number of individuals with advanced HIV disease[61,62,63] and lack of funding and infrastructure for routine viral load testing in low- to middle-income geographies, CD4 counts will continue to play an important role in managing HIV patients in terms of: stratifying long-term risk, fast-tracking onto ART outside the standard of care[64] and identifying patients with immunological or clinical failure.[60,65,66] The high number of individuals with advanced HIV disease also dictates that CD4 counts should play an important role in identifying patients at risk of opportunistic infections, such as cryptococcal disease, prophylactic treatment for tuberculosis and *Pneumocystis* pneumonia.

Limitations

Our study utilised samples mainly from high-income, industrialised or urban areas, and such as may not completely represent samples found in an entirely African population.

Conclusion

The AQUIOS PLG merges desirable aspects from conventional flow cytometer systems (high throughput, precision and accuracy, external quality assessment compatibility) with low technical skill requirements for automated, single platform systems.

Trustworthiness

The findings of these studies should be used per the scope of the study and with regard to the indicated limitations. The results review and release on AQUIOS should be performed by a qualified professional.

Acknowledgements

Data availability statement

All relevant data are within the article.

Disclaimer

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