

Research Article

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Assessment of EQA Reference Conventional Flow Cytometers for Clinical CD4+ T-Cell Enumeration

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ABSTRACT

Background: CD4+ T-cell enumeration continues to be an essential marker for assessing immunodeficiency and monitoring HIV disease progression. Enrollment in an external quality assessment (EQA) program is a strong tool for diagnostics as it allows for identification and correction of potential errors that may occur during testing. The aim of this article is to investigate the effect of our repeated participation in an EQA program on the performance of our internal flow cytometry instruments, namely the Navios and FACSCalibur, as an EQA provider.

Methods: We compiled data from 14 Canadian Immunology Quality Assurance Program (CIQAP) EQA sessions and compared the Navios and FACSCalibur results to the aggregate group mean (AGM) of each session. The standard deviation index (SDI), residual, and percent differential was performed to identify trends in data and identify any potential problems. A Bland-Altman analysis was performed comparing both of these flow cytometry instruments.

Results: Our results showed good performance overall with trend variation between the relative (%) and absolute count CD4+ T-cell results for both of the instruments. We observed a good agreement between instruments and identified that all HIV+ samples with low relative CD4+ T-cell results (<28%) were overestimated by Navios compared to FACSCalibur.

Conclusion: As an EQA coordinator, using the same instruments as participating laboratories allows more accurate monitoring of instrument performance and variability, supporting a stronger analysis of corrective action based on participant results.

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Background

In 2021, an estimated 38.4 million people were living with human immunodeficiency virus (HIV). Since the beginning of the HIV epidemic, 84.2 million people have been infected globally with more than 40.1 million deaths [1]. At the end of 2020, there was an estimated 62,790 people living with HIV in Canada [2]. CD4+ T-cell enumeration has been used extensively for decades to both identify patients in need of initiating antiretroviral therapy (ART) and to monitor those already on treatment [3]. Since 2015, the World Health Organization (WHO) has shifted to the use of plasma HIV viral load (VL) testing as the key indicator

for HIV-treatment monitoring [4]. However, the CD4+ T-cell enumeration method remains the primary approach to identify advanced immunosuppression (as indicated by a CD4+ T-cell absolute count <350 cells/ μ L) at the time of HIV diagnosis [5,6]. In addition, CD4+ T-cell enumeration continues to be an essential surrogate marker for assessing immunodeficiency and remains an integral part of follow-up monitoring in Canada [3,7].

Traditionally, CD4+ T-cell enumeration has been performed using flow cytometry, a technique that allows for detection of cells based on cell morphology, immunofluorescence of bound antibodies, and flow rate count with fluorospheres [8]. More recent technology includes point-of-care (POC) platforms which have been extensively adopted as an approach to overcome

challenges related to limited laboratory access and cost of reagents, particularly in developing countries with high HIV prevalence [9,10]. Conventional flow cytometry platforms remain the preferred technology used to perform clinical laboratory based CD4+ T-cell enumeration in many countries including Canada [11]. For these laboratories, enrollment in an external quality assessment (EQA) program plays an essential role in the reporting of accurate results and identification of potential errors [12].

The National Sexually Transmitted Blood-Borne Infection (STBBI) Laboratory Division within the Public Health Agency of Canada (PHAC) coordinates a National CD4+ T-cell enumeration EQA program called the Canadian Immunology Quality Assurance Program (CIQAP). This program was established in 1989 to improve the health of Canadians living with HIV/AIDS through the provision of a National Proficiency Testing Program for monitoring HIV disease progression. CIQAP participants are clinical laboratories across Canada using conventional flow cytometer platforms to analyze fresh human whole blood samples, either Beckman Coulter (BC) or Beckton Dickinson Biosciences (BD) instruments [11].

The conventional flow cytometry instruments are undergoing considerable evolution to make these instruments more cost-effective and user-friendly. These platforms use automated cell gating which adds considerable standardization benefits to flow cytometry for diagnostics [11]. CIQAP administration has integrated the same flow cytometry instruments used by the participants into the program to provide better instrument-specific support and assess remedial action cases specific to the CIQAP participants.

The aim of this article is to study the effect of repeated participation in the EQA program on our In-House (IH) BC Navios and BD FACSCalibur results performance. The relative (%) and absolute CD4+ T-cell results of these two instruments were compared to the results of the CIQAP aggregate group average (AGM). We also compared the overall agreement between these two conventional platforms. The data is based on results of 14 CIQAP EQA sessions for CD4 Tcell enumeration between 2016 and 2019.

Methods

CIQAP Panel Distribution, Evaluation and Reporting

Three times per calendar year, CIQAP participants across Canada receive quality control (QC) panels. Each panel consists of three fresh EDTA whole blood samples (24 - 48 hours old) from both HIV-positive and HIV-negative donors for proficiency testing and reporting of absolute and % T-cell enumeration for CD3, CD4, and CD8 results. Individual participant laboratory results are compared to the CIQAP AGM, a value that is based on the data input by all participating labs. The AGM value is calculated for both the % and absolute CD4+ T-cell enumeration results, excluding outliers that are outside of the ± 2 standard deviation index (SDI) acceptance range. A laboratory will be flagged for remedial action if the reported value has an SDI outside the ± 2.0 SD range, a residual value outside the ± 3.0 -point range for % CD4+ T-cell measurements, or a percent-differential value outside $\pm 15\%$ for absolute CD4+ T-cell counts when compared with the AGM. For these cases, CIQAP leads corrective action efforts to identify error sources and prevent recurrence.

CIQAP Internal Quality Evaluations

As part of CIQAP internal quality control procedures, the homogeneity and stability testing of CIQAP samples is evaluated

using statistical analyses in Annex B (normative): Homogeneity and stability of proficiency test items in ISO 13528, Statistical Methods for Use in Proficiency Testing by Interlaboratory Comparison. Additionally, the FACSCalibur and Navios systems include a built-in quality control system specific to each instrument. Both BD and BC manufacturers recommend running at least one type of control material. All test procedures were conducted following the manufacturer-provided protocols.

IH Navios and FACSCalibur Results Analysis

As part of the internal data assessment, all flow cytometry instruments are routinely compared to each other to assess agreement between different cytometry platforms and identify potential instrument-based biases that may skew the overall participant AGM. To evaluate IH Navios and FACSCalibur, flow cytometry printouts and electronic data files were compiled from 14 CIQAP sessions (from June 2016 to June 2019). The IH- Navios data was compared to the CIQAP overall AGM, as well as the AGM of only Navios users. Similarly, the IH FACSCalibur was compared to the CIQAP overall AGM to assess the performance of the instrument. Also, both instrument results were compared to one another to evaluate the overall agreement.

Sample Preparation and Gating Strategies

The analysis parameters for both platforms are based on a single platform method with a large panel of reagents and rigorous gating techniques, such as the PanLeucogating (PLG) method [13-16]. This PLG CD4 T-cell counting method uses a gating strategy based on the total number of white blood cells as a reference, rather than using the total lymphocyte population as recommended previously [13,17]. Samples were prepared as follows: a 100-mL aliquot of fresh whole blood was incubated with 20 μ L of BD MultiTest cocktail reagent (BD) containing CD3FITC/CD8PE/CD45PerCP/CD4APC or with 10 μ L of tetraCHROME CD45-FITC/CD4-PE/CD8-ECD/CD3-PC5 Antibody (BC) for 10 minutes at room temperature. BC instrument samples were then lysed using Immuno-Prep reagent (BC). Finally, 500mL of 2% paraformaldehyde (PFA) was added followed by 100 μ L of Flow-Count fluorospheres (BC).

Results

Instrument Usage in CIQAP Program

The conventional flow cytometers used by CIQAP participants include both BC and BD Biosciences instruments. BC instruments include the Navios, FC500, Aquios, and the Epics XL. BD Biosciences instruments include the FACSCanto/Canto II, FACSCalibur, and the FACSlyric. From 2016-2019, the distribution of the four most commonly used instruments during each session was analyzed. We observed a stable instrument distribution over time, with the exception of the Navios and FC500 (Figure 1). The prevalence of the Navios instrument has risen over this 4-year period. Initially, during the June 2016 session, the proportion of Navios users was 26.32% compared to 44.44% in June 2019, showing an increase by 18.12% (Figure 1). Concurrently, the number of FC500 users decreased as the number of Navios users increased. Of all the BD Biosciences instruments, we observed that the FACSCanto II is the most prevalent BD Biosciences instrument used in the CIQAP program and has been since 2016, with its percent distribution remaining relatively consistent at 38.89 - 45.16% (Figure 1). Though the Navios and the FACSCanto II are both conventional platforms, for the purposes of this study we focused on the Navios as it is the most commonly used instrument overall, making up almost 50% of all CIQAP instruments used since 2018.

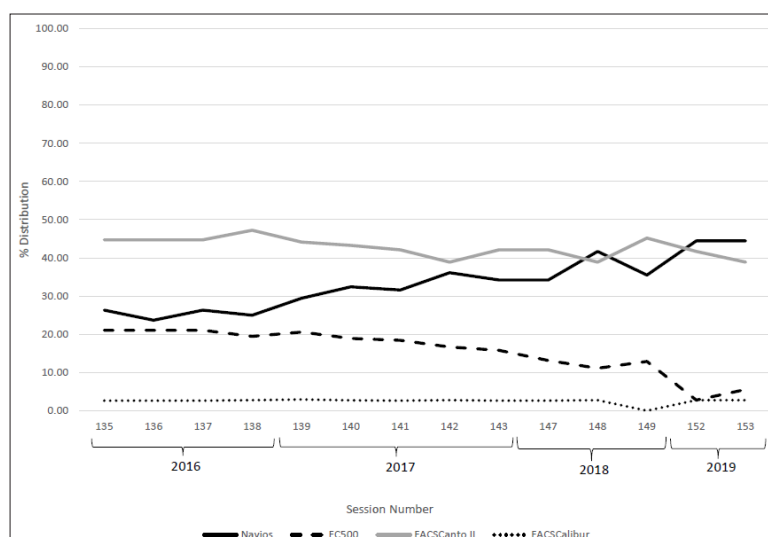


Figure 1: Beckman Coulter and BD Biosciences Main Instruments used by CIQAP Participants from 2016-2019

CIQAP Session Data Selection and Session Description

From June 2016 to June 2019, we identified 146 total participant responses and reports. During the 14 sessions, 40 proficiency samples were analyzed based on the CD4+ T-cell parameter, with a varying number of HIV+ and HIV- samples per year. An average of 6-8 cases per session required corrective action from 2016-2019 (Table 1). The normal range of CD4+ T cells in healthy adults has been established to be between 500 and 1,500 cells/ μ L for absolute count value and between 28% and 65% for % value [18, 19]. In terms of sample characteristics, there were 25 HIV+ CIQAP samples total; 8 samples had absolute CD4+ T-cell count <500 cells/ μ L and 17 samples with CD4+ T-cell count >500 cells/ μ L (Table 1).

Comparatively, there were 15 HIV- samples included, with 4 samples that had CD4+ T-cell count <500 cells/ μ L and 11 samples with CD4+ T-cell count >500 cells/ μ L. In terms of the CIQAP % CD4+ T-cell results, the range was 28.64% to 54.86% for all the HIV- samples and 8 HIV+ samples of the 25 had CD4+ T-cell % results below 28%.

Table 1: Data Subset General Information on CIQAP Participants and Sample Characteristics from June 2016 to June 2019. Changed from Total Remedial Action Cases in the Year to Average (Avge)/Session (Not Every Year Had the Same Number of Sessions Included Though, 2016-4 Sessions, 2017-5 Sessions, 2018-3 Sessions, 2019-2 Sessions; Sessions Only Included in Which Samples were Analyzed on Both the IH Navios and FACSCalibur.

Year	2016	2017	2018	2019	Sum
Number of Sessions (Avge)	4	5	3	2	14
Number of Participants (Avge)	38	37	35	36	146
Number of Samples (Avge)	12	15	9	6	42
HIV+ Samples	8	10	3	4	25
Samples <500 cells/mL	3	3	1	1	8
Samples >500 cells/mL	5	7	2	3	17
HIV- Samples	4	5	4	2	15
Samples <500 cells/mL	1	1	1	1	4
Samples >500 cells/mL	3	4	3	1	11
Number of Instruments (Avge)	38	37	35	36	146
Beckman Coulter	20	20	20	19	79
BD Biosciences	18	17	15	17	67
Number of Remedial Action cases (Avge/session)	8	6	7	6	27

Comparison of IH Navios and FACSCalibur Results to those Obtained from CIQAP Participants

The % and absolute CD4+ T-cell count results from the IH Navios and FACSCalibur were both individually compared to the CIQAP participants group mean. The residual analysis was performed for relative CD4+ T-cell results with an acceptance range of ± 3.0 points. For absolute CD4+ T-cell count results, percent differential analysis was performed with an acceptance range of $\pm 15\%$. Values found above and below the line of equality ($x=0$) are indicative of overestimation and underestimation respectively compared to the CIQAP AGM for both relative and absolute CD4+ T-cell counts. The line of equality ($x=0$) signifies no difference between the reported CD4+ T-cell enumeration results and the CIQAP AGM.

In Figure 2, residual and differential analysis of the IH Navios compared to the CIQAP AGM is shown, with the samples first arranged by HIV status then by increasing relative CD4+ T-cell results along the x-axis. We observed that only 3 samples were out of range in terms of CD4+ T-cell absolute count results and these samples were not specific to the group with low relative CD4+ T-cell levels (<28%) or HIV status. The samples that were outside of the acceptance range include samples D2 and C2 in Figure 2C and the sample E2 in Figure 2D. The percent differential results of these samples are 19.78%, 20.55% and 17.38% respectively, but in terms of the CD4+ T-cell SDI values, only the samples C2 and E2 were out of range, with SDI values of 4.90 for C2 and 3.71 for E2 (data not shown). Overall, both HIV+ and HIV- samples arranged by CD4+ T-cell absolute count results tend to be overestimated by the Navios (Figure 2C and D) contrary to the relative CD4+ T-cell results of both groups, which tends to be underestimated by the Navios (Figure 2A and B).

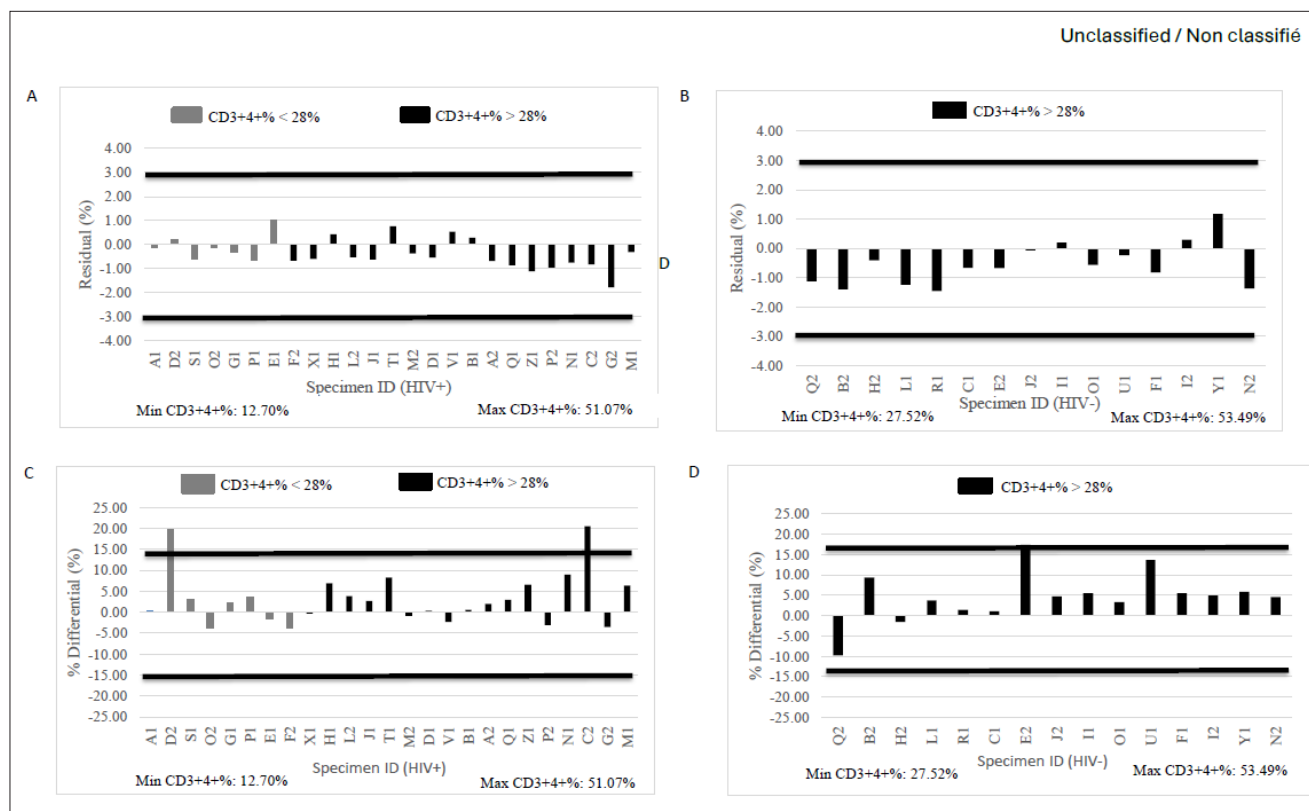


Figure 2: CD4+ T cell Residual and % Differential of Samples arranged first by HIV status (HIV+ and HIV-) then relative CD4+ T cell values of in-house Beckman Coulter Navios (from June 2016 to June 2019). (a) Residual analysis for HIV+ samples (CD3+4+% values). (b) Residual analysis for HIV- samples (CD3+4+% values). (c) Percent differential analysis for HIV+ samples (absolute CD3+4+ counts). (d) Percent differential analysis for HIV- samples (absolute CD3+4+ counts)

The relative and absolute CD4+ T-cell count results obtained on the FACSCalibur were compared to the CIQAP AGM in Figure 3. Residual and differential analysis of the IH FACSCalibur compared to the CIQAP AGM is arranged in the same manner as figure 2. The IH FACSCalibur exhibited a similar trend to the IH Navios, except in the case of relative CD4+ T-cell results for HIV+ samples (Figure 3A). The IH FACSCalibur does not show the same tendency to underestimate relative CD4+ T-cell results when compared to the CIQAP AGM. Also, it can be noted that the samples D2, C2, and E2 are not out of range in terms of the percent differential results on the IH FACSCalibur in comparison to the Navios.

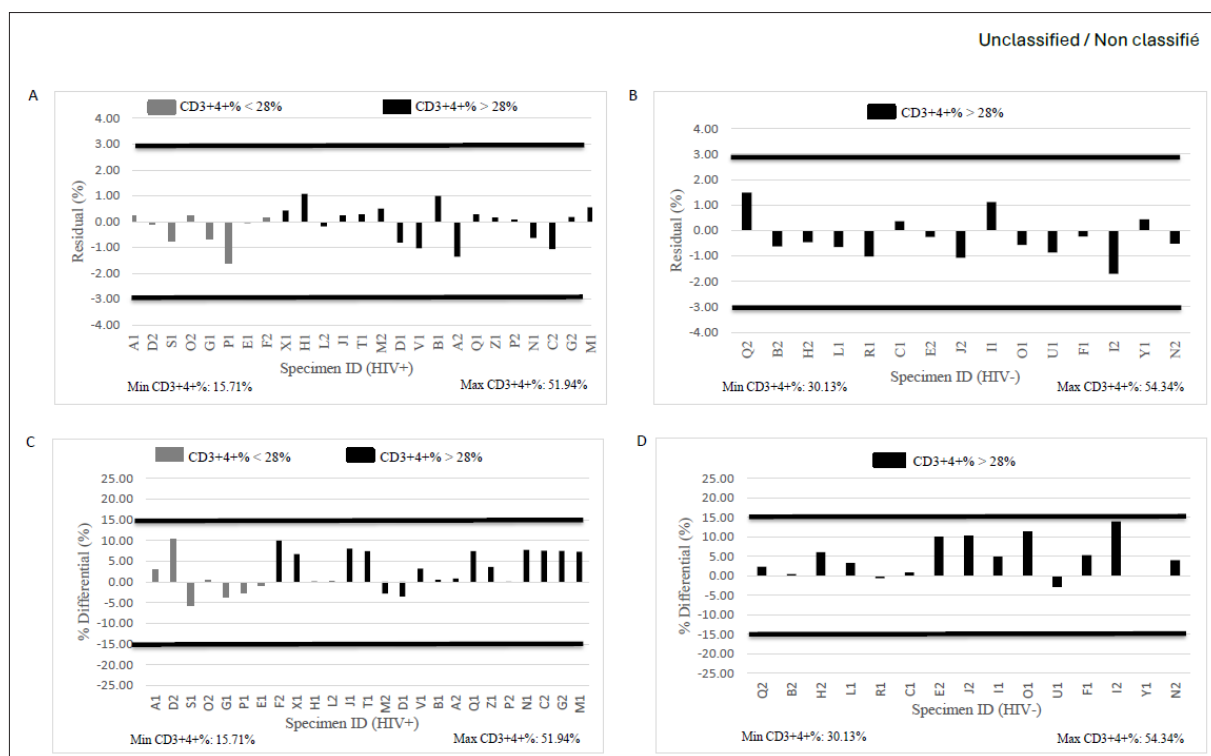


Figure 3: CD4+ T cell Residual and % Differential of Samples arranged first by HIV status (HIV+ and HIV-) then relative CD4+ T cell values of in-house BD Biosciences FACSCalibur (from June 2016 to June 2019). (a) Residual analysis for HIV+ samples (CD3+4+% values). (b) Residual analysis for HIV- samples (CD3+4+% values). (c) Percent differential analysis for HIV+ samples (absolute CD3+4+ counts). (d) Percent differential analysis for HIV- samples (absolute CD3+4+ counts).

To ensure that there was no inherent instrument bias within the AGM calculations, analysis was performed with the IH Navios compared to the AGM calculated from Navios users only, as well as the CIQAP AGM excluding the Navios users, with samples arranged in the same way. This analysis showed the same previously observed trends when compared to the CIQAP AGM (data not shown).

Evaluation of IH Navios Results in Samples with CD4 T-Cell Counts > 500 and < 500 Cells/ μ L

The IH Navios was further evaluated, with samples arranged depending on the absolute CD4+ T-cell count threshold (ie. < 500 or > 500 cells/ μ L). The 3 samples C2, D2 and E2 that were out of range are not specific to a group of cells count threshold (ie. < 500 or > 500 cells/ μ L). The samples C2 and D2 have absolute values greater than 500, unlike E2 which has an absolute value less than 500 (data not shown). As in the case of the HIV status results analysis, we also noted a difference in terms of the trend between the relative and percent differential CD4+ T-cell results. The IH Navios consistently underestimates relative CD4+ T-cell results but overestimates absolute CD4+ T-cell counts when compared to the CIQAP AGM, irrespective of the CD4+ T-cell counts being < or > 500 cells/ μ L (data not shown).

Analysis of Remedial Action Needs of the IH Navios Instrument

Overall, there were 27 cases recorded during this 4-year period (Table 1) and the most common problem identified with Navios user's platform was an analysis issue (gating and acquisition). However, the remedial action analyses identified pre-analysis problems for the three out of range IH Navios samples. Specifically, pipetting precision was the source of the IH Navios failure for samples C2, D2, and E2 (data not shown).

Comparison of CIQAP IH Instrument Evaluations

As part of the routine operational analysis of CIQAP's IH instruments and data, a Bland Altman analysis was performed for the IH Navios versus the IH FACSCalibur. The goal is to evaluate the bias in the mean differences between the sample results acquired on both instruments and assess the degree of agreement between the Navios results and the FACSCalibur results, which acts as the reference instrument. To quantify agreement between the two instruments, statistical limits of agreement are calculated using the mean and the standard deviation of the differences between two measurements. The resulting XY scatter plot shows every difference between the two instruments plotted against the mean difference between the two instruments (bias). The limits of agreement were determined such that 95% of the data points lie within ± 1.96 standard deviation units [20]. If the line of equality ($x=0$) was not contained within the confidence interval of the bias, this indicated a substantial systematic difference in which the IH Navios instrument consistently over- or under- estimates compared to the IH FACSCalibur.

In Figure 4, we observed that the bias results above the mean are representative of overestimation by the IH Navios and bias results found below the mean are representative of underestimation by the IH Navios, compared to the IH FACSCalibur. Values are bound by the 95% agreement limits (± 1.96 standard deviation), with outliers found outside of this range. We identified two distinct populations:

samples with low (<28%) CD4+ T-cell relative results that tend to be overestimated by the Navios and samples with mid- to high (>28%) relative CD4+ T-cell results that tend to have equal distribution above and below the mean bias (-0.42) (Figure 4).

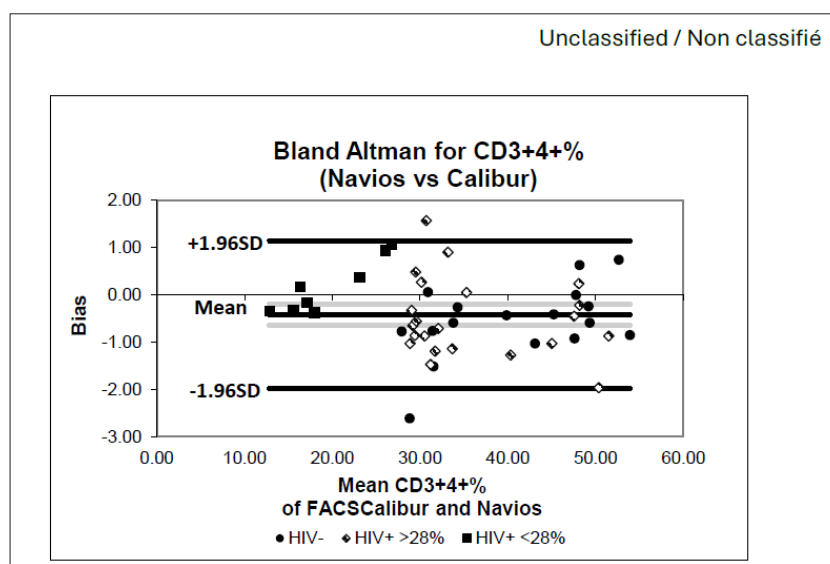


Figure 4: In-house Beckman Coulter Navios versus in-house BD Biosciences FACSCalibur Bland Altman analysis plot for % CD4+ T cell enumeration (CD3+4+%) results from June 2016 to June 2019 CIQAP results (14 sessions). In-house Beckman Coulter Navios versus BD Biosciences FACSCalibur Bland Altman analysis of all samples.

We analyzed the eight samples with low relative CD4+ T-cell counts. Although they had low CD4+ percentages, they had an absolute CD4+ T-cell count below 500 cells/ μ L, with the exception of G1 (23.36%; 698 cells/ μ L) and E1 (27.32%; 509 cells/ μ L).

Discussion

In this study, we, as a CD4+ T-cell enumeration EQA provider, evaluated the performance of flow cytometer instruments the: IH Navios and FACSCalibur during various EQA sessions.

These two instruments generally demonstrated good performance throughout the different EQA sessions compared to the group of participants. Indeed, across the 14 EQA sessions included in this study, the results obtained were generally close to the group average. Only one EQA session result with the IH Navios instrument required corrective action analysis, due to insufficient performance related to the enumeration result of the absolute value of CD4+ T cells. This underperformance resulted from a lack of precision in the pipetting technique during sample preparation, a problem attributable to the operator rather than the instrument. Analysis of the trends of these two IH platforms relative to the group average across of samples (HIV+ vs. HIV-) showed that both instruments behaved similarly for absolute CD4+ T-cell counts, but not for CD4+ T-cell percentages. Indeed, in terms of percentage results for CD4+ T cells, the IH Navios instrument underestimated HIV+ samples with a high percentage of CD4+ cells (>28%) compared to the CIQAP group average. However, the FACSCalibur instrument showed an equal distribution for both HIV+ and HIV- samples. It is likely that the different reagents used for these instruments, and how they interact on a biochemical level with the cells, could explain this observation.

Sustained high performance depends on both the rigorous application of good laboratory practices and regular, proper instrument maintenance. Overall, the observed performance was consistent with that of other participants or clinical laboratories evaluated. In addition to the individual evaluation of these two IH instruments compared to the CIQAP program participant group, we also introduced a comparative approach between the two instruments. The results showed a good agreement between them. As an EQA

coordinator, employing the same instruments as the participants plays a crucial role in ensuring reliability and remains essential for rigorous monitoring of our practices, which is essential for maintaining a high level of quality assurance. It helps to strengthen the accuracy of CD4+ T-cell enumeration measurements, while promoting transparency, trust, and continuous improvement in program management. Furthermore, given the evolution of flow cytometry technology toward increasingly automated systems, it is essential for an EQA provider to have robust resources enabling in-depth evaluation of complex sample analysis cases. This ensures better support for operators facing potential analytical errors.

The support we provide to the flow cytometry operators is of paramount importance, particularly when analyzing complex samples, but also in terms of providing tailored advice to optimize instrumental parameters, correctly identify relevant cell populations, and recognize potential artifacts. This expertise strengthens the reliability of results, the reproducibility of analyses, and improved monitoring of treatment for people living with HIV.

The performance of these IH instruments was not assessed for HIV+ samples with low CD4+ T-cell counts (< 350 cells/ μ L) as these results are often observed in patients that have developed immune deficiencies due to HIV. The CIQAP program uses whole blood samples that are representative of HIV+ samples analyzed in Canadian clinical laboratories. The majority of these samples have absolute CD4+ T-cell count values > 400 cells/ μ L because therapeutic management intervention is initiated very early after diagnosis, despite cell counts. This consideration of local specificity on the QC sample profile of the program ensures the accuracy of diagnoses and treatments. It also provides an advantage by allowing participants to detect errors and inconsistencies in their results, thus offering opportunities for continuous improvement of laboratory practices.

The analyses carried out in this study helps us to continuously monitor the performance of our instruments and thus contributes to maintaining the quality and credibility of our program. It also allows us to implement effective monitoring of internal validation processes for the QC equipment used (homogeneity and stability

testing), enabling quality monitoring of our program and providing robust tools to better assess the performance of our participants. Likewise, it is important to have tools to identify best practices for quality service, including monitoring and adjusting the analysis methods used by our participants, ensuring consistent and comparable results, and making the group average more representative. This avoids disparities in measurement methods, which could lead to variations not due to biological differences between patients, but rather to the instruments themselves. This significantly improves the quality of the assessment, the accuracy of the data, and the ability to make reliable clinical interpretations to ensure the validity and rigor of the program.

Conclusion

Overall, this study demonstrated that the IH Navios and FACSCalibur are both reliable and accurate instruments operating within the CIQAP program. They are used as monitoring controls for the stability of the EQA samples, which provides us with tools for a more thorough analysis of participant results. In HIV infection treatment, CD4 T-cell enumeration using conventional flow cytometry instruments is still critical to help guide ART initiation and patient care in tandem with viral load testing [21].

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Human Ethics and Consent to Participate Declarations:

The Office of Intellectual Property Management and Business Development (OIPMBD) of the National Microbiology Laboratory approved the full study. We confirm that this study follows the OIPMBD guidelines and regulations, and informed consent was obtained from all participants.

Consent to Publish declaration missing: it's not applicable because the manuscript contains no identifying information or data from individual persons.

Data Availability: The datasets generated and/or analysed during the current study are not publicly available for CONFIDENTIALITY reasons but are available from the corresponding author on reasonable request.

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