Evaluating New Biomarkers for Epstein Barr Virus Infection in Egypt

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ABSTRACT:

Some reports have suggested EBV to be a trigger agent for an autoimmune hepatitis, even EBV has been suspected as a probable cause of particular granulomas in the liver and even in a rare vanishing bile duct syndrome. This study aimed to evaluate $CD19^+$, $CD23^+$ and $CD24^+$ B- lymphocytes as biomarkers for EBV early diagnosis in EBV-liver disease patients. ***Subjects &*** *Fifty subjects included in this work, grouped into: control group and EBV mono-infected patients' group. Immunophenotyping in peripheral blood samples was performed for $CD19^+$, $CD23^+$ and $CD24^+$ B-cells using flow cytometry technique. $CD23^+$ B-cells frequency was the most significant (P <0.001) changed CD marker- among the estimated CDs- in EBV group versus control group (49.43±0.58 vs 21.18±1.10), respectively. Both $CD19^+$ and $CD23^+$ B-cells frequency correlate strongly with liver transaminases. Diagnostic performance of this panel of CD markers was obtained by applying receiver operating characteristic (ROC) curve analysis; $CD23^+$ B-cell frequency was the most precise CD marker- of this panel- in distinguishing EBV cases from controls. The AUC of $CD23^+$ B-cell frequency was 0.998, with sensitivity= 96% and specificity= 100% at cut-off value of 44.4%. Its NPV was ultimate (100%). On the other hand, $CD19^+$ B-cells frequency did not show any response as diagnostic tool, and $CD24^+$ B-cells frequency showed very little response in this context. $CD23^+$ B-cell frequency has a strong correlation with liver transaminases which may mirrored the severity of the disease. Additionally, it may represent a promising co-biomarker with the currently used EBV- viral capsid antigen (EBV-VCA) antibodies for early EBV diagnosis.*

Key words: *EBV, early diagnosis, $CD19^+$, $CD23^+$, $CD24^+$, Flow cytometry.*

1. INTRODUCTION:

Epstein-Barr virus (EBV) is a ubiquitous gamma herpes virus with tropism for B cells. It is the most common persistent virus infection in humans with approximately 95% of the world's population showing an asymptomatic life-long carrier status. This sustained life-long latent infection is the result of the unique interaction of EBV with B cells, specifically memory B cells, which are the EBV reservoir in healthy individuals. The disruption of this finely regulated balance between virus and host immune system can result in EBV-associated lymphoproliferations (LPD) of B, T and NK cell derivation [**Dojcinov *et al.*, 2018**].

Primary infection of EBV is usually asymptomatic and occurs early in life. When symptomatic, it is usually a self-limited disease occurring in adolescents or young adults manifested as acute infectious mononucleosis (IM) [**Rostgaard *et al.*,**

2019]. The liver is frequently affected by EBV infection; in more than 90% of cases of EBV-related mononucleosis, liver involvement is present **[Schechter and Lamps, 2018]**. Moreover, EBV infection underlies the development of hematologic (*i.e.*, Burkitt and Hodgkin lymphoma) and epithelial (*i.e.*, nasopharyngeal carcinoma) tumors [**Mawson and Suvankar, 2017**]. Additionally, it was being as well associated to some autoimmune disorders (*e.g.*, Multiple Sclerosis) [**Guan *et al.*, 2019**].

Conventional diagnosis of EBV depends on EBV-VCA for IgM and IgG, but recent studies had shown high false positive rates for these antibodies [**Elansary *et al.*, 2016**]. Cluster of differentiation (CD) molecules are surface molecules expressed on cells of the immune system that play key roles in immune cell-cell communication and sensing the microenvironment. These molecules are essential markers for the identification and isolation of leukocytes and lymphocyte subsets [**Kalina *et al.*, 2019**].

Some CDs were reported to be tightly reflecting B cells condition, those are $CD19^+$, $CD23^+$ and $CD24^+$ B cells [**Delage *et al.*, 2019**]. $CD19^+$B cell level was estimated in patients with anti-EBVCA IgM positivity [**Luo *et al.*, 1991**]. CD23-known as Fc epsilon RII, or FcεRII- is a B cell activation marker, proved to be closely associated with EBV infection [**Selb *et al.*, 2017**]. CD23 expression was subsequently shown to be required for EBV induced immortalization [**Kang** and **Kieff, 2015**]. In addition, $CD24^+$- known as signal transducer CD24 or heat stable antigen CD24 (HSA) is a cell adhesion molecule, was found to be expressed at the surface of most B lymphocytes and differentiating neuroblasts [**Daniel *et al.*, 2016**].

Going with this line, the present work aimed to investigate other modalities as $CD19^+$-, $CD23^+$- and $CD24^+$- B lymphocytes in early EBV mono-infected Egyptian patients, that can improve early diagnosis of EBV.

2. SUBJECTS & METHODS:

2.1 Study design:

This case-control study was applied on 50 subjects, divided into 2 groups (control and EBV mono-infected patients). All patients were selected from outpatients of the "Internal Medicine" clinic, Alexandria University Hospital, Alexandria city- Egypt, through January 2015 to January 2016. Apparently healthy individuals were driven from blood donors and acted as control group. All of subjects were from the same governorate, but had different occupations. The ethical approval of the faculty was performed on the study protocol, and enrolment of the individuals to the study was conditioned by an informed consent.

The control group included 25 apparently healthy subjects, while the EBV group included 25 newly diagnosed patients. Both groups were matched in age and gender. All enrolled subjects were subjected to full medical history, and complete clinical examination. Diagnosis of EBV was based on clinical examination, laboratory tests, as well as ultrasonography in some cases.

2.2 Exclusion Criteria:

Patients were selected to have no history of cardiovascular disease or diabetes mellitus, recent drug intake (of any kind) at least 14 days before sampling, and also negative for: serum HCV-RNA, HBs-Ag, cytomegalovirus (CMV), autoimmune hepatitis, and sclerosing cholangitis. Women undergoing pregnancy and lactation, and patients with underlying diseases requiring long-time corticosteroids or immunosuppressive treatments were also excluded from this study.

2.3 Laboratory Analyses:

Serum and whole EDTA-blood samples were collected *via* venipuncture the median cubital vein in closed sterile tubes. Any hemolyzed, icteric, or turbid sample was avoided. All samples involved in this study were subjected to duplicate analyses.

2.3.1 Hematological and biochemical analyses:

total leucocytic count and hemoglobin were measured using (Siemens- Advia 2120i, Germany), liver enzymes: alanine transaminase (ALT) and aspartate transaminase (AST) were measured by commercial kits using UV-visible spectrophotometer (Thermo Spectronic Unicam Helios Alpha, UK).

2.3.2 Serological analysis for EBV infection:

The diagnosis of primary EBV-hepatitis was made based on both: positive immunoglobulin M antibody to EBV viral capsid antigen (anti-EBV-VCA) IgM and Epstein-Barr virus EA IgG. It was accomplished by using (Robonik Read Well Stirp ELIZA Analyzer) apparatus, and kits had been used were of catalog numbers "ESR1361G" and "ESR1363G" from SERION ELISA classic, Germany. The results of EBV IgM and IgG measurements were expressed as optical density (450nm). All the selected patients were reactive to EBV-VCA IgM, indicating acute infection.

2.3.3 Immunophenotyping $CD19^+$, $CD23^+$, and $CD24^+$ B-lymphocytes:

For quantification of the percentage of B-cell for CD 19^+, 23^+ and 24^+, "Ficoll-Diatrizoate" density gradient solution for isolation of lymphocytes (#824012: Bio-Rad, Hercules, CA, USA) had been used for isolation of lymphocytes from diluted blood samples onto the density gradient. Then lymphocytes from the blood samples were prepared and fixed in a 1.0% (w/v) solution of paraformaldehyde was prepared by dissolving paraformaldehyde in phosphate buffered saline (PBS) at 70°C and sterile filtration through a 0.45-pm filter. The pH of the solution was confirmed to be 7.4, then samples were preserved in 4°C until analysis [**Lal *et al*., 1988]**.

Identification of B-cell subpopulation's percentage in whole blood was carried out using flow cytometry technique. A region (gate) was defined around total lymphocytes in peripheral blood, depending upon cell size (forward scatter) and complexity (side scatter). The sample is incubated with fluorochrome labeled monoclonal antibodies that are specific for cell surface antigens: $CD19^+$, $CD23^+$, and $CD24^+$ (Recombinant Anti-CD19 antibody [BU12] (ab254170), APC Anti-CD23 antibody [2G8] (ab25457), and Biotin Anti-CD24 antibody [30-F1] (ab25215): abcam-USA). Then, analyzed on a flow cytometer; cells that have bound the labeled antibodies can be quantified on the bases of their fluorescence emission. Light scatter gating for the expected lymphocyte region is carried out using linear forward angle (FSC) and side light scatter (SSC). Manufacturer's instructions were strictly followed up and the results were expressed as percentages.

2.3 **Statistical Analysis:**

Statistical package for the social sciences (SPSS) computer program-version 22- was used (IBM SPSS Statistics for Windows, Armonk, NY) to analyze the results. Data were illustrated as mean± standard error (M±SE) and independent sample t-test was performed for comparing quantitative variables between the two-tested groups. Bivariate correlation between the estimated CD markers and the other parameters was performed too. Receiver operating characteristic (ROC) curve analysis was applied to detect the potentiality of each CD measured in this

work as a diagnostic tool for EBV at best cutoff value for each. *P*- value <0.05 was considered statistically significant.

3.RESULTS:

3.1 Subjects' characteristics & laboratory analyses studies:

Collectively, 50 subjects involved in this work, consisting of 22 men (44%) and 28 women (56%), aged between 31-52 years (M±SE= 41.60±0.75). Demographic, haematological and biochemical results are shown in table (1).

3.2 Flow cytometry analyses:

Resulted flow cytometry analyses for $CD19^+$, $CD23^+$, and $CD24^+$- B lymphocytes frequencies- in both groups- is illustrated in the following figure (1). While the precise measured frequencies for the estimated CDs are illustrated in underneath table (2).

3.3 Correlation between tested CD markers and other parameters:

Pearson's correlation was performed to get the correlations between the parametric variants (frequencies of $CD19^+$, $CD23^+$, and $CD24^+$ B-lymphocytes) and the other investigated laboratory analyzed parameters among the whole tested cohort, as shown in table (3).

3.4 Diagnostic performance of investigated CDs in EBV:

ROC curve analysis was performed to examine the potentiality of each of $CD19^+$, $CD23^+$, and $CD24^+$ B-lymphocytes in differentiating EBV patients from apparently healthy controls as illustrated in figure (2). Comparison with each other concerning: AUC, sensitivity, specificity, 95% confidence interval (95% CI), positive and negative predictive values, in addition to significance of the test are all shown in the underneath table (4).

Detailed frequency-estimation of the three-tested CD surface markers were performed by subclassifying their results into weak-, moderate- and high- positivity in each group, as illustrated in the following table (5):

4.DISCUSSION:

Immunophenotyping is used in the diagnosis of EBV infection- as symptoms of EBV infection are like other illnesses- and expected to open a new era in the field of EBV disease research and EBV disease diagnostics [**Arai, 2019**]. Thus, this paper attempts to investigate the variant frequency of $CD19^+$, $CD23^+$ and $CD24^+$ markers in EBV patients, and to examine their potentiality in diagnosing early EBV infection.

Table (1) shows that age and gender were non-significantly changed between groups. TLC was significantly elevated (*P*<0.001) in EBV group compared to the control group (M±SE: 11.12±0.77 *vs* 6.66±0.30), respectively. This result was in harmony with other studies that noticed elevation in TLC in about 40-70% of patients with EBV [**Yang *et al.*, 2014; Rochford**, **2016**]. It was attributed to a complex interaction between virally encoded proteins and B-cell specific cellular proteins which constitute the proliferation-inducing program [**Shrivastava *et al.*, 2016**]. In contrast, Hgb level did not show significant change between the two groups in our study; and **Zhang and coauthors** [**2019**] were in agree with this result.

ALT and AST (table: 1) were significantly increased (*P*<0.001) in EBV group compared to the control group (M±SE: 32.20±1.34 *vs* 15.48±0.64 for ALT), and (M±SE: 28.24±1.14 *vs* 14.72±0.72 for AST), respectively. EBV should considered as a possible causative agent for this elevation if there was no other reason for acute hepatitis [**Yang *et al.*, 2014; Rochford**, **2016**]. In spite of being within normal range, this condition of slight elevation of liver transaminases was

explained by a study made by **Fugl and Andersen [2019]** who found that liver enzymes peaked within 14 days and returned to normal after the first presentation of EBV infection such as fever, sore throat, or lymph node swelling.

Concerning CD- surface markers (table: 2), quantification of $CD19^+$ frequency revealed a significant (P<0.001) decrease in the EBV group compared to the control group (27.64±0.75 *vs* 48.15±1.05), respectively. This was in concordance with other studies performed by **Shoman *et al.*, [2014] and Luo *et al.*, [2018]**. Recently, it was also indicated that more than half (56%) of the EBV patients had decreased number of $CD19^+$ B-cells compared with healthy individuals in United States, which augmented our results too **[Chatterjee *et al.*, 2019**]. In contrary, **Luo and coworkers** in **1991** were against these findings; they demonstrate elevation in frequency of $CD19^+$ B cells in the EBV patients. $CD24^+$ was also decreased significantly (P<0.001) in the EBV group *vs* the control group (20.94±0.52 *vs* 27.59±0.33), respectively. Other studies had the same results as ours, and attributed it to the cross-linking of $CD24^+$ with apoptosis in $CD24^+$ B-lymphocyte cells [**Kondo *et al.*, 2011; Tsai *et al.* 2017**].

On the other hand, $CD23^+$- B lymphocyte cells were the only tested-CD member that significantly (P<0.001) increased in EBV patient's group compared with the control group (49.43±0.58 *vs* 21.18±1.10), respectively as shown in table (2). This result was in harmony with other studies [**Luo *et al.*, 1991; Pan *et al.*, 2014; Selb *et al.*, 2017**]; which referred this result to the stimulatory role of $CD23^+$ in EBV-transformation of B-cells. Investigations had shown that only cells co-expressing Epstein-Barr nuclear antigens and $CD23^+$ undergo immortalization [**Mühe and Wang, 2017; Yap *et al.*, 2019**]. Additional study applied by **Mrozek-Gorska and coauthors [2019]** supported our result too, as $CD23^+$ was one of certain activation markers ($CD23^+$, $CD27^+$, $CD38^+$) that were induced by viral infected B cell.

Correlation between each of tested CD- surface marker frequency and the investigated laboratory parameters (table: 3) was done. Pearson's correlation revealed that $CD19^+$ had a strong negative correlation with ALT (r=-.859, P=0.01), AST (r=-.830, P=0.01), and fair negative correlation with TLC (r=-.539, P=0.01) at the 2-tailed significance level. $CD23^+$ was also strongly positively correlated with ALT (r=.838, P=0.01), with AST (r=.822, P=0.01), and fair positive correlation with TLC (r=517, P=0.01) at the 2-tailed significance level. No correlations had been obtained for $CD24^+$ B-lymphocytes frequency with any of the estimated laboratory parameters.

Early in this year **[2019]**, **Yap *et al.*** mentioned that atypical cell expressed-$CD23^+$ was positive for confirming the EBV diagnosis. So, diagnostic efficacy for each tested CD marker was examined by performing ROC curve analysis (figure: 2). It showed a promising role for $CD23^+$ far greater than $CD24^+$, while $CD19^+$ did not show such role (table: 4). It has a sensitivity and specificity (96%, 100%), respectively, at cut off value of 44.4%. It covers almost all the AUC (0.998), possesses ultimate NPV (100%) and 96.2% PPV, with a high significance value (P<0.001).

The unexpected results for $CD19^+$ and $CD24^+$ in ROC curve analysis recruited us to subclassify the frequencies of both into three grades after **Subramanian and coauthors [2005**] (as shown in table: 5). It obviates that: for $CD19^+$, all the control members were in the moderate- and high-positive grade, while all the EBV group members laid in the weak-positive grade. This may explain the zero response of $CD19^+$ as an EBV- diagnostic tool. For $CD24^+$-very weak response for ROC curve study (AUC= 0.026, with sensitivity= 4%, and specificity= 36%, as shown in table: 4),

it may be attributed to that all tested subjects had the same weak-positive grade (control and EBV patients).

5.CONCLUSION:

Overall, these findings may nominate cellular $CD23^+$ to play a role as a complementary biomarker in early diagnosis of EBV, together with the conventionally used EBV–VCA antibodies.

6.RECOMMENDATIONS:

Correlation between these CD-surface markers and more clinicopathologic features in EBV- associated liver disease is recommended.

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Table (1): Demographic, haematological and biochemical characteristics.

Variable	Control group (n=25)	EBV group (n=25)	*P*
Age: M±SE (Range)	40.72±1.01 (31-50)	42.48±1.10 (33-52)	N.S.
Gender: Female (%) Male (%)	13(52%) 12(48%)	10(40%) 15(60%)	N.S.
TLC ($1\ 0^3/mm^3$) (Range)	6.66±0.30 (4.10-9.10)	11.12±0.77 (5.30-17.8)	<0.001
Hgb (gm/dL) (Range)	12.90±0.20 (11.50-15.40)	12.76±0.27 (10.10-15.10)	N.S.
ALT (U/L) (Range)	15.48±0.64 (10-21)	32.20±1.34 (22-44)	<0.001
AST (U/L) (Range)	14.72±0.72 (10-25)	28.24±1.14 (20-39)	<0.001

All data are expressed as: M±SE; TLC: total leucocytic count; Hgb: haemoglobin; ALT: alanine transami-nase, AST: aspartate transaminase.

Table (2): Frequencies of the investigated CD- surface markers.

Variable	Control group (n=25)	EBV group (n=25)	*P*
$CD19^+$ (%): Mean± SE (Range)	48.15±1.05 (40.00-55.40)	27.64±0.75 (22.70-33.10)	<0.001
$CD23^+$ (%): Mean± SE (Range)	21.18±1.10 (16.10-42.10)	49.43±0.58 (41.50-54.20)	<0.001
$CD24^+$ (%): Mean± SE (Range)	27.59±0.33 (24.50-29.90)	20.94±0.52 (16.70-28.40)	<0.001

CD: cluster of differentiation; *P*: significance.

Table (3): Pearson's correlation between tested-CD markers and investigated parameters.

Variable	$CD19^+$		$CD23^+$		$CD24^+$	
	r	***P***	***r***	***P***	***r***	***P***
ALT	-0.859	0.01	0.838	0.01	-	-
AST	-0.830	0.01	0.822	0.01	-	-
TLC	-0.539	0.01	0.517	0.01	-	-

Correlation is significant at the 0.01 level (2-tailed); *r*: correlation; *P*: significance.

Table (4): Diagnostic efficacy of the estimated CD- surface markers for EBV.

Marker	AUC	Cut-off Value	Sensitivity	Specificity	95%-CI	PPV	NPV	*P*
$CD19^+$ (frequency)	0.000	-	-	-	-	-	-	-
$CD23^+$ (frequency)	0.998	44.4%	96%	100%	0.993-1.000	96.2%	100%	<0.001
$CD24^+$ (frequency)	0.026	28.35%	4%	36%	0.000-0.076	60%	90%	N.S.

CD makers are represented in % of circulating B-lymphocytic cells; PPV: positive predictive value; NPV: negative predictive value.

Table (5): Grades of each tested-CD in both groups.

CD Marker		Control group (n=25)	EBV group (n=25)	*P*
$CD19^+$%: Weak-positive	(up to 35%)	0(0%)	25(0%)	<0.001
Moderate-positive	(35-50%)	15(60%)	0(0%)	<0.001
High-positive	(>50%)	10(40%)	0(0%)	<0.001
$CD23^+$%: Weak-positive	(up to 35%)	24(96%)	0(0%)	<0.001
Moderate-positive	(35-50%)	1(4%)	15(60%)	<0.001
High-positive	(>50%)	0(0%)	10(40%)	<0.001
$CD24^+$%: Weak-positive	(up to 35%)	25(0%)	25(0%)	N.S.
Moderate-positive	(35-50%)	0(0%)	0(0%)	N.S.
High-positive	(>50%)	0(0%)	0(0%)	N.S.

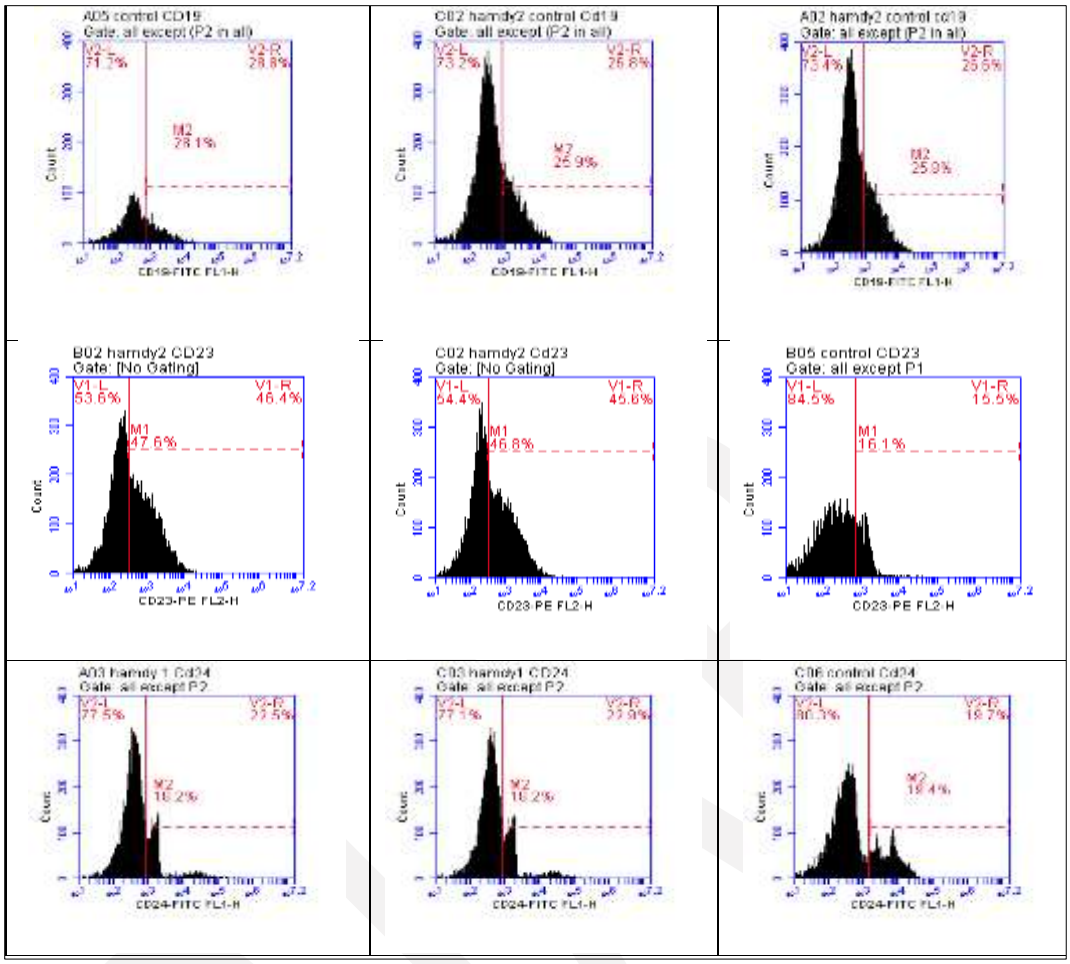

Fig. (1): Performance of flow cytometry on peripheral blood of participated subjects to estimate $CD19^{+}$-, $CD23^{+}$, and $CD24^{+}$- B lymphocytes percentages; M2: positive population.

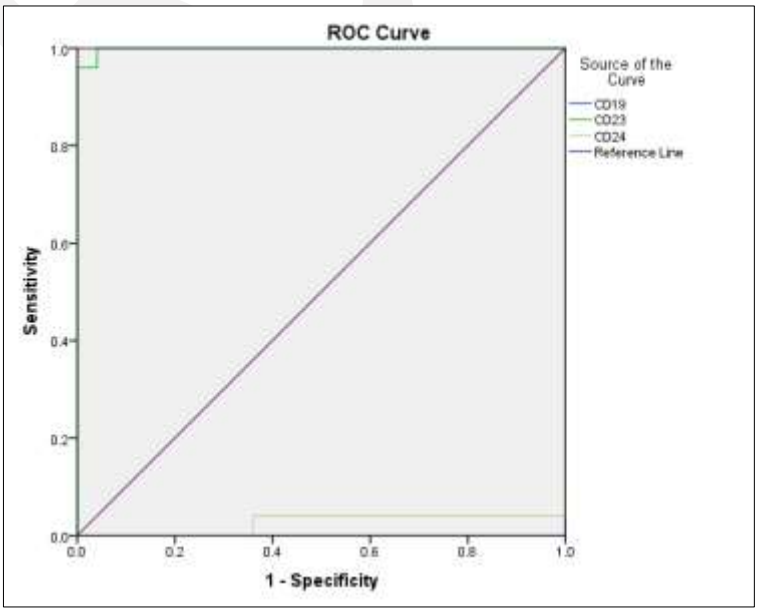

Fig. (2): Diagnostic performance of the estimated CDs