

Importance of Finding Circulating Tumour Cells in Breast Cancer Patients' Peripheral Blood: Creating a Workable Method

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ABSTRACT

Background. Circulating tumor cells (CTCs) Originating from the primary tumor and disseminating via the circulatory system. The abundance of circulating tumor cells is positively linked to metastatic progression, and this increase is inversely Correlated with the survival outcomes of breast cancer patients and provides more accurate information about patient-specific diagnoses. Therefore, Detection of CTCs with high sensitivity in peripheral blood can function as a reliable tool for cancer diagnosis in the context of liquid biopsy. **Objective.** This study developed an accurate technique for detecting CTCs in a single milliliter of peripheral blood. **Materials and Methods.** Eighty women with different clinicopathological data and 20 matched healthy volunteers were included. To identify CTCs *via* a qRT-PCR assay, cytokeratin-14 and 19 and vimentin were selected as target genes. A FACS assay was performed with antibodies against cytokeratin-14, 16, 19, vimentin, and CD45. For the spiking experiment, MDA-MB-231 cells and HCT 116 cells were used to validate both methods. **The results.** Vimentin is a crucial marker for the identification of CTCs *via* either “qPCR-RT” or “FCAS”, followed by cytokeratins. A statistical Assessment of the qRT-PCR data showed A notable positive relationship between cytokeratin-14 and vimentin expression. The clinical stage of disease was significantly negatively correlated with cytokeratin-19 and positively correlated with vimentin expression. The statistical analysis of the FACS data revealed A meaningful positive linkage between clinical stage and either ^{VIM+}CTCs or ^{VIM+CK+}CTCs. **Conclusion.** The qRT-PCR assay was more accurate and sensitive than the FACS assay for Identification of CTCs within peripheral blood samples with high performance. Due to the heterogeneity and variability of breast carcinoma, the study recommended using different cytokeratins and vimentin antibodies to assess the disease, especially in the early stages.

Keywords: Breast Cancer, Circulation Tumor Cells, qRT-PCR, Flow Cytometry, Cytokeratins, Vimentin

INTRODUCTION

The worldwide cancer scenario is changing, with breast cancer (BC) has emerged as the most frequently occurring carcinoma on a global scale, overtaking lung cancer, based on the data published by the International Agency for Research on Cancer (IARC) in December 2020. Breast cancer constitutes 1 in 8 cancer diagnoses across the globe, Representing 2.3 million incident cases in males and females combined [1]. Breast cancer constitutes 18.9% of the overall cancer burden in Egypt (32.04% in women and 2.2% in men), corresponding to an age-standardized incidence rate of 49.6 per 100,000. [2]. BC disease is multifactorial and caused by a variety of conditions [3]; therefore, its classification is very useful for diagnosing, determining the prognosis, and developing Non-hazardous practices and creating focused breast cancer screening initiatives [4], whereas treatment can be highly effective when the disease is identified early [5]. Metastasis involves a sequential process wherein cancer cells form A primary tumor can infiltrate adjacent tissues, enter the bloodstream or lymphatic system as circulating tumor cells (CTCs), and eventually colonize and proliferate in distant organs [6]. Thomas Ashworth (1869) conducted the initial examination of circulating tumor cells (CTCs) and demonstrated that the “CTCs” Within the circulatory system, which are identical to those of the tumor itself, may provide insights into the origin of multiple tumors in a single individual [7]. Before they enter the circulatory system, tumor cells undergo a variety

of phenotypic and genotypic alterations, such as changes in cytoskeletal structure, the secretion of proteases, and modifications in the expression profiles of adhesive proteins and receptors. These transformations enable CTCs to adopt an invasive phenotype and separate from the primary tumor [8]. After detaching from the original tumor, tumor cells located near newly formed blood vessels infiltrate blood vessels through the loosely arranged vascular endothelium, aided by increased pressure from tumor growth, which promotes their extrusion [9]. Consequently, CTCs could be beneficial for screening, assessing patient prognosis, and predicting responses to therapy [10]. The detection of CTCs is anticipated to serve as a noninvasive method for tumor screening in clinical settings, capturing the diverse nature of tumors [7]. Moreover, this detection technique is safer and more straightforward compared to traditional needle biopsies, which carry risks such as bleeding and infections [11]. Nonetheless, Technical challenges arise in detecting these cells due to the minimal concentration of CTCs in preperitoneal blood [7], ranging from 1 to 100 cells per millilitre [12], their brief half-life, and the lack of precise surface markers [13]. Consequently, high specificity and strong affinity are essential for their successful capture. As a result, there has been increasing interest in the clinical use of CTC detection, particularly During the early course of breast cancer. Typically, the process of CTC detection consists of several steps: capture, isolation,

enrichment, and detection [14, 15]. Numerous factors can alter the physical and Intrinsic biological traits of CTCs, including the abnormal growth and metabolism of tumor cells, changes in cell composition, altered gene expression and modification, and the production and buildup of polar particulates. Given these changes, scientists have developed technologies Aimed at enriching, isolating, and profiling CTCs by their physical and biological transformations [7]. Given that CTCs are rare cancer cells and provide A sophisticated detection system, suitable techniques and equipment are essential for identifying these cells in low quantities within blood, as roughly one CTC can be found per milliliter of blood escaping from primary tumors or metastases. A widely used approach in CTC research involves identifying CTCs in whole blood through quantitative real-time polymerase chain reaction (qRT–PCR) [7]. Additionally, analysis of live CTCs by flow cytometry is another high-throughput method that has been extended by various research teams to CTC analysis [16]. Cytokeratins (CKs) are gold-standard markers for detecting CTCs and are found only in epithelial cells during the epithelial-mesenchymal transition (EMT) stage [13]. Additionally, Vimentin expression is regarded as a hallmark of EMT associated with tumorigenesis, is a component of the cytoskeleton, and is crucial for the conversion of a normal cell into an aggressive tumor cell [17, 18]. According to research on The pattern of vimentin and cytokeratin expression in breast cancers, Their coexpression in breast cancer cells is associated with phenotypic plasticity and increased metastatic capacity [19]. Therefore, the present study used two different techniques, qRT–PCR and flow cytometry (FACS), to establish a sensitive and specific technique for detecting “potential” CTCs from just one millilitre of blood. These two methods are more effective for early diagnosis and may aid in recurrence prediction and follow-up to increase survival.

Subjects and Methods

Blood Samples

The present study involved 80 women with clinically confirmed breast cancer, who were further categorized into subgroups based on their clinicopathological data. To establish a control group, 20 healthy female donors, matched by age and without any previous history of malignancy, were also recruited. All participants underwent a clinical examination, The study excluded individuals undergoing chemotherapy or radiotherapy. Prior to study participation, all individuals voluntarily signed a written informed consent form, consistent with the Ethical Guidelines of the Medical Research Institute, Alexandria University (Appendix 1 for the Informed Written Consent for Patient Participation in Clinical Research, 2011). Peripheral blood samples were collected in 5-mL EDTA-K2 Vacutainer tubes. The initial few milliliters were discarded to prevent endothelial cell contamination during venipuncture [20]. For molecular investigations, 1 ml of EDTA-treated blood was used to measure the cytokeratin-14, 19 (CK-14, 19) and vimentin (VIM) gene expression. For cytological investigations, 1 ml of fresh EDTA blood was used for the detection of CTC numbers via FACS using CKs cocktail: CK-14, CK15, CK16, and CK-19, VIM, and CD45 combined with propidium iodide (PI) to select viable cells [13].

Cell Lines

MDA-MB-231 breast cancer (mesenchymal-like; HTB-26™) and HCT116 colon cancer (ATCC-CCL-247) cell lines were used for spiking experiments and obtained from the American Type Culture Collection

(ATCC). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin, and incubated at 37°C in a CO₂ incubator after which contamination was regularly checked during growth using a standard protocol.

Cell Spiking

The spiked cells were used for the validation of the analytical specificity, sensitivity, and recovery rate of the qRT-PCR and FACS techniques. In addition, they were used to determine the analytical accuracy, efficiency, and reproducibility of CTCs detection methods using CKs and VIM. MDA-MB-231 cells Served as a positive control for vimentin, while HCT116 cells were used as a positive control for CKs. Both cell lines were CD45 negative. After the Cell culture was performed in accordance with ATCC protocols, the cell suspensions were collected using the trypsin treatment Cell viability was assessed by trypan blue dye exclusion and counted with a hemocytometer; the percentage of viable cells was greater than 98%. Cells were adjusted to a concentration of 10,000 cells/mL in 1x PBS (10 cells/μL). For MDA-MB-231 cells, <5, 5, 10, 20, 40, and 80 cells were spiked/1 ml blood, while for HCT116 cells, <5, 10, 13, 25, 50, and 100 were spiked/1 ml blood. The cells were counted under a microscope at 4x magnification, and the number of cells was noted in each drop. One milliliter of dilution buffer was used to rinse the chamber, after which the cells were allowed to flow through the blood sample. The clean chamber was subsequently checked under a microscope, after which the remaining Cells were enumerated, and the quantity of spiked cells was determined by adding all the counts from every drop and then deducting the number of cells that remained on the slide. Three replicates of each cell line were individually micropipetted and spiked into each tube of blood according to the number needed.

Detection of Potential CTCs using Real-Time Assay “qRT-PCR”

Total RNA was isolated from patients with BC, healthy donors, spiked cells, and cell lines, as controls, following the guidelines provided by the easy-REDTM Total RNA Extraction Kit (iNtRON Biotechnology, Inc.). All procedures for RNA extraction were carried out in a laminar flow hood, maintaining RNase-free conditions. The concentrations of RNA and their contamination ratios were Quantified by measuring absorbance at 260/280 nm using a NanoDrop spectrophotometer. (Jennway Nanodrop, USA).

The integrity of the RNA was evaluated through PCR amplification of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene was performed. Complementary DNA (cDNA) was synthesized from 5 μg of total RNA using the SuperScript III First Strand Synthesis Super Mix Kit, following the manufacturer's instructions (Invitrogen, Darmstadt, GER). Each run included a negative control without RNA and a positive control that contained RNA sourced from cell lines. Primers for the target genes were designed and tested via an NCBI Nucleotide Blast. CK-14,19 and VIM were designed as the main markers for identifying potential CTCs in blood. Additionally, GAPDH was used as an endogenous control. Table 1 The reaction tubes were prepared according to the instructions of the TOPreal™ qPCR 2X PreMIX Kit (iNtRON Biotechnology, Inc.). Each PCR reaction contained 2 μL of RT product, 200 nM of forward and reverse primers, 10 μL of SYBR Green master mix, and nuclease-free water to a final volume of 20 μL. Reactions were initially incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s,

and 72 °C for 30 s using an Applied Biosystems 7500 instrument. Each amplification run included negative and positive controls.

Target gene expression was quantified as relative quantity (RQ), where the amount of mRNA in the tumor samples was normalized to

that of the endogenous control (GAPDH) and subsequently normalized to that of the control samples via the equation $RQ = 2^{-\Delta\Delta C_t}$. $\Delta\Delta C_t = [(C_{t_{\text{interest gene}}} - C_{t_{\text{endogenous control}}})_{\text{Sample}} - (C_{t_{\text{interest gene}}} - C_{t_{\text{endogenous control}}})_{\text{Control}}]$.

Table 1: Sequences of the Primers used in this Study and their Accession Numbers

Primers	Forward (5' → 3')	Reverse (5' → 3')	Access.No
CK-14	GAAGGATGCTGAAGCCTGGT	GTCAGTAACCTCGGACTGC	NM_000526.5
CK-19	CCAGTTCTCCTCTGGATCGC	TCCAGTGGGATCTGTGTCCA	NM_002276.5
VIM	GACAATGCGTCTCTGGCAGCTCTT	TCTTCTGCCTCTGCAGGTTCTT	NM_003380.5
GAPDH	GAAGGTGAAGTCTGGAGT	GAAGATGGTGTGGGATTTTC	NM_001357943

Validation of the “qRT-PCR” Assay

The National Center for Biotechnology Information (NCBI) Nucleotide Blast was used to design and validate the specificity of the primers, and the results were validated via OligoArchitect design to determine the ability of the primers to form duplexes (www.OligoArchitect.com). The dissociation curves were used for the determination of the specificity of primers and the assay. Previously, QRT-PCR was performed, the RNA concentration was determined, and the purity ratio (1.8-2.0) was checked. Additionally, the specificity of the qRT-PCR assays was validated using control reactions lacking reverse transcriptase (no-RT control) to assess potential DNA contamination. Following optimization, assay performance parameters were evaluated, including accuracy, efficiency, dynamic range, linearity (R^2), limit of detection (LOD), limit of quantitation (LOQ), and precision (RDsr).

Detection of Potential “CTCs” using Flow Cytometry Assay

The traditional three-step staining method, which takes more than two hours, has been streamlined to a two-step procedure that takes less than 45 minutes. Consequently, PI dye was applied to stain the sample surfaces simultaneously, resulting in sections that tested negative for CD45 while showing positivity for intracellular markers, such as VIM and CKs. This approach is efficient and specific, enabling rapid counting of possible CTCs using established markers for Epithelial cells analyzed using a direct intracellular flow cytometry assay (13). This was a fifth-step process. The first step involved staining for the CD45 cell surface marker; 5 µl of APC-Cy7-labelled mouse anti-human antibody against CD45 (BD Biosciences) Was mixed with 1 mL of peripheral blood, vortexed thoroughly, and incubated for 25 min at room temperature (RT) in the dark. In the second step, Cells were fixed with 2% freshly prepared cold paraformaldehyde in PBS and incubated at room temperature in the dark for 15 min with gentle agitation. The third step involved lysis of red blood cells (RBCs); 1x BD FACS lysis solution was added to the cells, following a 20 min incubation at RT in the dark, samples were centrifuged at 1500 rpm for 5 min. The supernatant was discarded, leaving the pellet intact. Permeabilization was achieved by resuspending the pellet in 200 µL of 0.1% Triton X-100 for 10 min at 25 °C. The fifth step was intracellular staining; Alexa Fluor 488 conjugated mouse anti-human vimentin (BD Bioscience) and Alexa Fluor 647 conjugated mouse anti-human CKs cocktail (CK-14, 15, 16, 19) (BD Bioscience) were added to the cell pellet, vortexed well, Cells were incubated for 30 min at room temperature in the dark, then washed once with 1 mL PBS and centrifuged at 1500 rpm for 5 min. The supernatant was carefully removed, and the pellet was resuspended in 0.5 mL sheath buffer before analysis on a BD FACSC system (BD Biosciences) software. Fluorescence quantification was conducted through FACS utilizing the staining index (SI). The SI was determined using the following formula: (mean fluorescence intensity

of the positive signal – mean fluorescence intensity of the negative control) / ($2 \times$ standard deviation of the mean fluorescence intensity of the negative control). An initial gate was employed to select large events; subsequently, it was plotted against propidium iodide (PI) to isolate only nucleated cells. Debris was eliminated, and any remaining contaminating CD45+ Cells were omitted from the analysis. Supplementary figure 1.

Validation of the FACS Assay

To ensure the specificity of our measurements, A range of internal quality assurance procedures was implemented, including daily calibration of the instrument's optics, fluidics, and electronics. Fluorescence compensation and detector voltages were adjusted using BD CS&T beads (BD Biosciences). Additionally, Antibodies employed in combination were confirmed for specificity on cell lines expressing the relevant markers using FACS analysis, this allowed confirmation of their specificity and compatibility with the FACS preparation procedure. HCT166, MDA-MB-231, and leukocyte cells served as controls to confirm the sensitivity of the antibodies. The HCT166 colon cancer epithelial-like cells acted as a positive control for CKs, while leukocytes served as a negative control. VIM expression was assessed in MDA-MB-231 breast cancer mesenchymal-like cells as a positive control and in HCT166 cells as a negative control. CD45 was assessed in leukocytes as a positive control and in HCT116 cells as a negative control. The sensitivity of the antibodies was assessed by spiking known numbers of cells into 1 ml of peripheral blood as follows: <5, 5, 10, 20, 40, and 80 MDA-MB-231 cells were spiked into the blood samples from six healthy donor. Similarly, <5, 10, 13, 25, 50, and 100 HCT116 cells were spiked into 6 blood samples from healthy subjects. Once the assay had been optimized, the most sensitive conditions were identified: accuracy, linearity, LOD, LOQ, and RDsr.

Statistical Analyses

Statistical analyses were conducted with SPSS, version 21. The data are expressed as the mean $\pm$ standard error (S.E.). The count data are expressed as percentages. Additionally, the measurement data among groups were compared with the Mann–Whitney (U) test. The ROC curves were plotted and analysed for each assay to determine the diagnostic accuracy at specific cut-off values and for the sensitivity and specificity of the test. $p < 0.05$ Was regarded as indicative of a statistically significant difference. Finally, the correlations between the parameters were analysed by a suitable correlation test.

Results

The clinicopathological features of BC patients were summarized in the Table 2. The mean age of the healthy women (50 years; range from 40-57 years) was matched with the mean age of the BC patients (53 years; range from 38-58 years).

Table 2: Clinicopathological Features of BC Patients

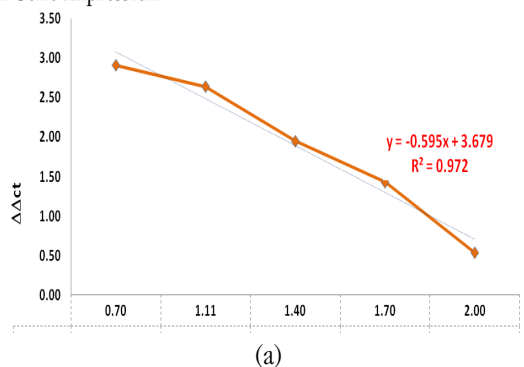
Clinicopathological Features	No. Women (N=80)	Frequency (%)
Clinical Stage		
I	13	16.25
II	31	38.75
III	36	45
Histological Grade		
II	65	81.25
III	15	18.75
Axillary Lymph Node Involvement		
Positive	67	83.8
Negative	13	16.2
Estrogen Receptor (ER) Status		
Positive	50	62.5
Negative	30	37.5
Progesterone Receptor (PR) Status		
Positive	50	62.5
Negative	30	37.5
Her-2 Expression		
Positive	70	87.5
Negative	10	12.5

Detection of Potential “CTCs” using qRT–PCR

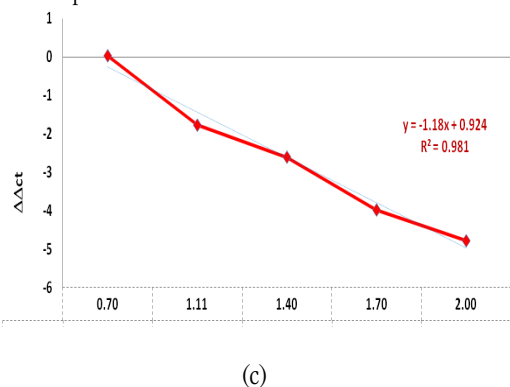
Validation of the “qRT–PCR” Assay

The CK-14 and 19 genes were used as epithelial markers, while the VIM gene was used as a mesenchymal marker to identify CTCs in the peripheral blood *via* qRT–PCR. Data from the NCBI and OligoArchitect servers showed that (a) the primers were homologous to the desired target sequence, (b) the primers and amplicon did not implement a secondary structure (Self-3” complementary ≤ 3 score), (c) Indicated a low likelihood of oligonucleotide self-hybridization in the reaction (second complementary ≤ 6 score), and (d) all primers used had Comparable melting temperatures (T_m difference $\leq 5^\circ\text{C}$) and did not form strong 3’-duplexes ($\Delta G \geq -2.0$ kcal).

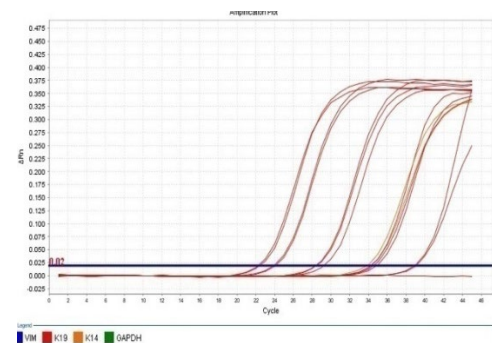
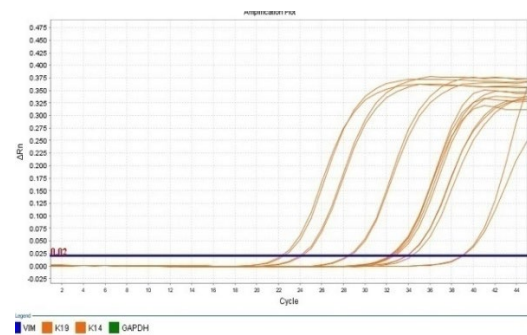
CK14 Gene Expression



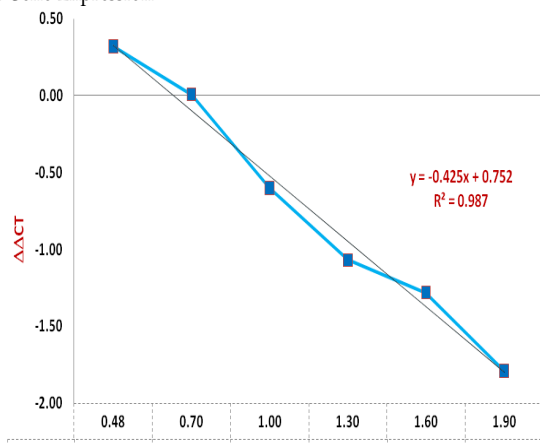
CK19 Gene Expression



These findings confirmed the primer specificity. The concentrations of the isolated RNA were measured, and the purity ratios ranged from 1.8 to 2.1 before qRT–PCR. Additionally, during the qRT–PCR assay, melting curves for the target genes showed a sharp peak corresponding to the specific product at $> 80^\circ\text{C}$, with very little nonspecific product at lower temperatures. Additionally, negative controls did not amplify the genes. These observations confirmed the assay sensitivity. Moreover, the assay sensitivity and performance were measured *via* recovery curve compared of known numbers of spiked cells / 1 ml of blood for both cell lines. This wide range of cells was tested, allowing determination of the dynamic range of the technical assay. Fig.1



VIM Gene Expression



(e)

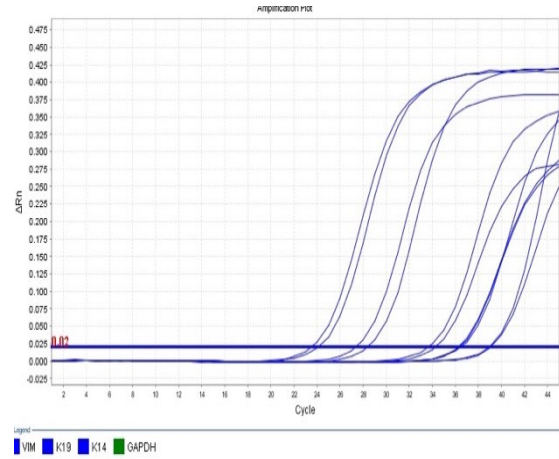
(f) MDA-MB-231 Cells (Positive Control);
Dynamic Range from (<5 to 80 cells)/ 1ml Blood

Figure 1: Recovery Curves of (a) CK14, (c) CK19 and (e) VIM Genes. Determination of Assay Efficiency was Performed by Plotting the $\log_{10}$ of the Number of Spiked Cells Against the $\Delta\Delta Ct$. High Reproducibility and Wide Range of Detection using known Numbers of Spiked Cells/1 ml of Blood ((b) CK14, (d) CK19 and (f) VIM). Three Replicates were Run for each Sample, and Amplification of the Target Gene was Detected using SYBR Green Dye.

Gene Expression Analysis

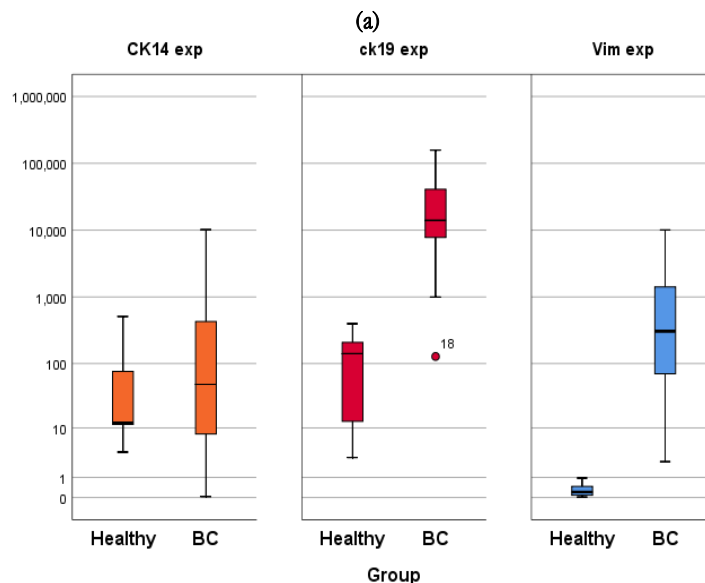
Analysis revealed a significant elevation of CK14 expression in breast cancer patients compared with healthy controls ($p < 0.05$). Expression was notably higher in TNBC than non-TNBC women, while clinical stage did not significantly affect CK14 levels. Statistical analysis showed that CK-19 Expression levels were significantly elevated in women with BC than in healthy women ($p < 0.05$). There was no significant difference between TNBC and NTNBC patients. However, CK-19 expression was significantly lower in patients with clinical stage III disease compared to those with stage I or II. The

analysis of VIM expression Women with BC exhibited significantly increased expression than in healthy women. Its expression was also significantly higher in TNBC compared to its expression in NTNBC patients. Although VIM expression was elevated in patients with clinical stage III compared to those in stages I and II, the observed difference did not achieve statistical significance. (Fig. 2) A significant positive association was found between CK14 and VIM expressions and between clinical stage and VIM expression, as well as between clinical stage and VIM expression; conversely, a negative correlation between clinical stage and CK-19 expression. Table 3

Table 3: Correlation Analysis of qRT-PCR between the Studied Parameters

		CK14	CK-19	VIM	Stage
CK-14	r_s	1.000	-0.277	0.648**	0.255
	P	.	0.101	0.00**	0.120
CK-19	r_s		1.000	-0.234	-0.501**
	P		.	0.135	0.006**
VIM	r_s			1.000	0.366*
	P			.	0.039*

r_s Spearman's rho **Correlation was Significant at the 0.01 level, * Correlation was Significant at the 0.05 Level



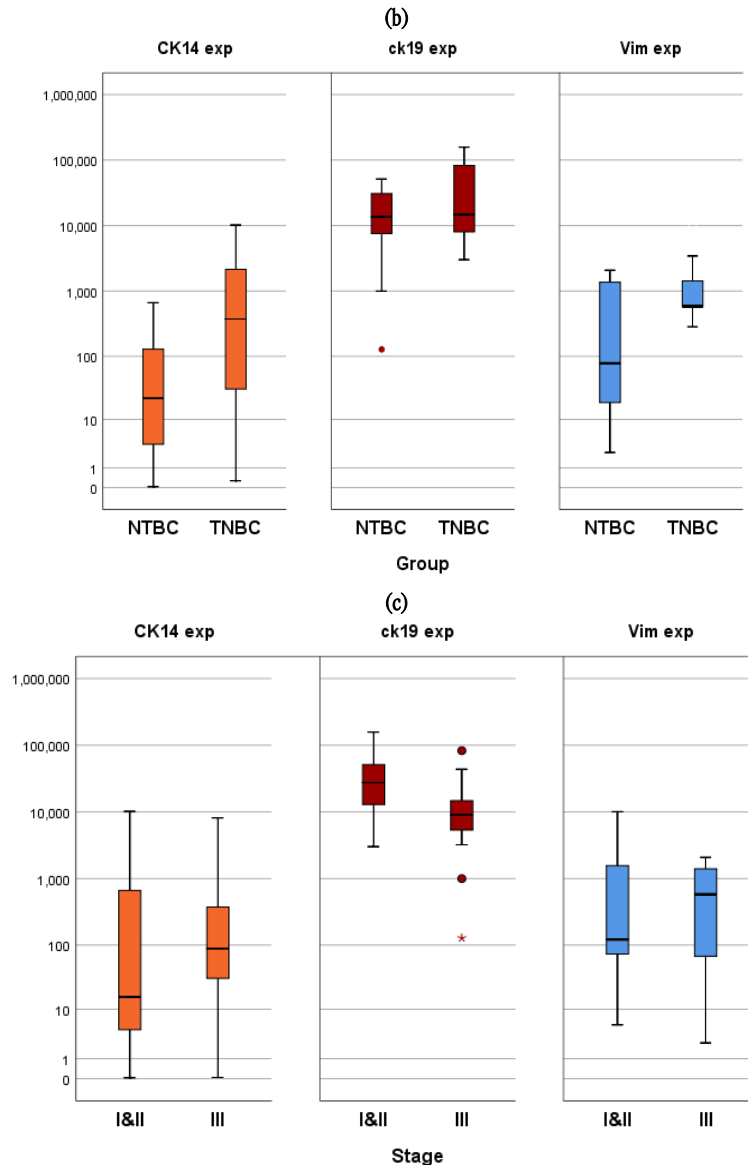


FIGURE 2: Box Plots of the Statistical Analysis of CK-14, CK-19 and VIM Expression in Patients with (a) BC and Healthy Controls, (b) NTNBC and TNBC Patients and (c) Clinical Stages I-II and III of BC

Receiver Operating Characteristic (ROC) Curve Analysis

The accuracy of CK-19 expression for identifying BC was notably high at $95 \pm 0.039\%$, Yielding a sensitivity of 87% and specificity of 83% at a cut-off of 395 copies per 2 μ l cDNA. Additionally, VIM expression exhibited an exceptional accuracy of 100%, along with 100% sensitivity and specificity at a cut-off value of 1.7 copies/2 μ l cDNA. Conversely, CK-14 expression did not show significant accuracy. For identifying the TNBC subtype, CK-14 expression was determined to be an effective marker with an accuracy of $69.4 \pm 0.108\%$, a sensitivity of 55.6%, and a specificity of 85% at a cut-off value of 268.2 copies/2 μ l cDNA. In

comparison, CK-19 expression had an accuracy of $74.4 \pm 0.102\%$, with a sensitivity of 44.4% and a specificity of 80% at a cut-off value of 14659 copies/2 μ l cDNA. The accuracy of VIM expression reached $87.2 \pm 0.067\%$, featuring a sensitivity of 55.6% and a specificity of 90% at a cut-off value of 1381 copies/2 μ l cDNA.

When identifying late clinical stage III, VIM expression showed an accuracy of $77.4 \pm 0.092\%$, with a sensitivity of 77% and a specificity of 81.3% at a cut-off value of 235 copies/2 μ l cDNA. In contrast, there were no significant differences observed in CK-14 and CK-19 expressions. Table 4 and Fig 3.

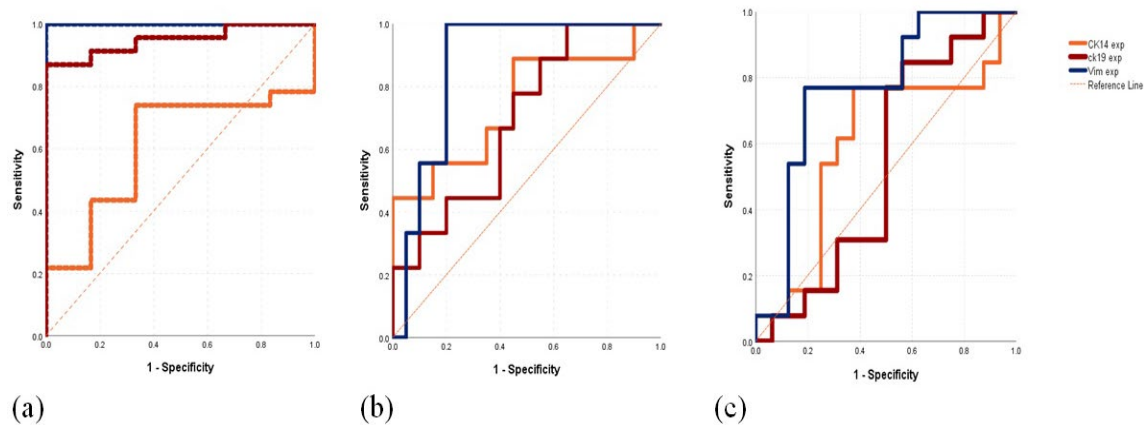
Table 4(a): Diagnostic Test, qRT-PCR, of CK-14, 19 and VIM

Gnes	Healthy women (n = 10)	BC Women (n = 80)	Cut off	Sensitivity	Specificity	PPV %	NPV %	Accuracy $\pm$ S.E	P-values
CK-14	(100%)	(100%)	82.0	43.5	83.3	43	75	61 ± 0.117	0.378
CK-19	(100%)	(100%)	395	87	83.3	84	88	95 ± 0.039	$< 0.001^{**}$
VIM	(100%)	(100%)	1.7	100	100	92	100	100 ± 0.0	$< 0.001^{**}$

Table 4(b): Diagnostic Test, qRT-PCR, of CK-14, 19 and VIM

Gnes	Healthy women (n = 10)	BC Women (n = 80)	Cut off	Sensitivity	Specificity	PPV %	NPV %	Accuracy± S.E	P-values
CK-14	(100%)	(100%)	268.2	55.6	85	56	64	69.4±0.108	<0.033*
CK-19	(100%)	(100%)	14659	44.4	80	90	20	74.4±0.102	0.05*
VIM	(100%)	(100%)	1381	55.6	90	100	14	87.2±0.067	<0.002**
Genes	Stage I&II Women (n = 37)	Stage III Women (n = 33)	Cut off	Sensitivity	Specificity	PPV %	NPV %	Accuracy ±S.E	P-values
CK-14	(100%)	(100%)	82.0	53.8	75	54	70	59.6±0.133	0.318
CK-19	(100%)	(100%)	13858	30.8	68.8	77	9	53.4±0.111	0.759
VIM	(100%)	(100%)	235	77	81.3	92	10	77.4±0.092	0.016*

** Correlations were Significant at the 0.01 Level., * Correlations were Significant at the 0.05 Level., PPV: Positive Predictive Value, NPV: Negative Predictive Value

**FIGURE 3:** Diagnostic Accuracy of (a) CK-14, (b) CK-19 and (c) VIM Expression Levels for Identifying (a) BC, (b) TNBC and (c) Clinical Stages I-II and III using qRT-PCR Assay.

Detection of Potential “CTCs” using Flow Cytometry

Validation of the FACS Assay

HCT116 cells exhibit universal spread and cytoplasmic expression of cytokeratins, while leucocytes exhibit negative expression of the protein. VIM was diffused inside the cytoplasm of MDA-MB231 cells, while leucocytes were very weakly stained. On the other hand, HCT116 cells were not stained. The leukocyte cell surface was the only site at which CD45 was labelled. Conclusively, the antibodies applied were specific and suitable for use in the FACS preparation process. The results indicated that gating performed better with a smaller number of cells compared to a larger quantity.

This result can be attributed to the reduced time needed to pick and spike fewer cells, which reduces cell mortality while promoting cell recovery; the recovery rates of spiked cells exhibited a robust linear relationship, with correlation coefficients (R^2) of 0.895 for VIM and 0.92 for CKs. These findings were utilized to confirm sensitivity.

Detection of Potential “CTCs” using Flow Cytometry Assay

CK⁺CTCs and VIM⁺CTCs were detected in women with BC but not in healthy donors. CK⁺CTCs were significantly lower in TNBC than in NTNBC patients. Additionally, their numbers in clinical stage III patients were significantly lower compared to those in clinical stages I and II. The number of VIM⁺CTCs was significantly higher in TNBC patients compared with NTNBC patients. Although counts were elevated in clinical stage III patients relative to stages I and II, the difference did not reach statistical significance. Moreover, the statistical analysis of cells that expressed both CKs and VIM proteins, CK⁺VIM⁺CTCs, showed that their numbers were significantly higher in TNBC than in NTNBC patients. Additionally, A significant increase in their counts was observed in clinical stage III relative to stages I and II. Fig. 4 Correlation analysis revealed a significantly strong direct correlation between clinical stages and VIM alone (VIM⁺CTCs) or between clinical stages and cells expressing both CK and VIM proteins, CK⁺VIM⁺CTCs. Table. 5

Table 5: Correlation Analysis, FACS Assay, between the Studied Parameters

		CK+ CTC	Vim+CTC	Vim+CK+CTC	Stages
CK+CTCs	r_s	1.000	0.082	-0.132	0.057
	P	.	0.328	0.235	0.380
Vim+CTCs	r_s		1.000	0.766**	0.679**
	P		.	0.00**	0.00**
Vim+CK+CTCs	r_s			1.000	0.534**
	P			.	0.001**

r_s : Spearman's Correlation, ** Correlations were Significant at the 0.01 Level (1-tailed).

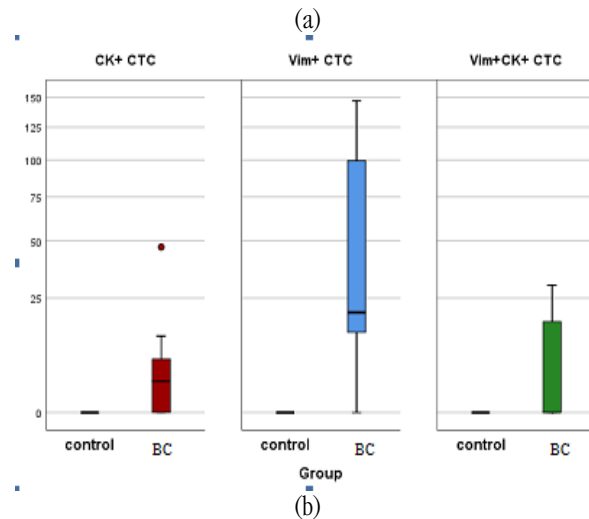


Figure 4: Descriptive Analysis of (a) CK⁺CTCs, (b) VIM⁺CTCs and (c) VIM⁺CK⁺CTCs Numbers to Distinguish between (a) BC and Healthy Female Patients, (b) NTNBC and TNBC Patients, and (c) Clinical Stage of Breast Cancer.

Receiver Operating Characteristic (ROC) Curve Analysis

For the identification of BC disease, CK⁺CTCs displayed significant accuracy, $77 \pm 0.08\%$, Demonstrating a sensitivity of 54% and specificity of 100% at a threshold of 1.5 cells. The VIM⁺CTCs analysis demonstrated notable accuracy, $98 \pm 0.02\%$, with a sensitivity of 96% and a specificity of 100% at a threshold value of 1 cell.

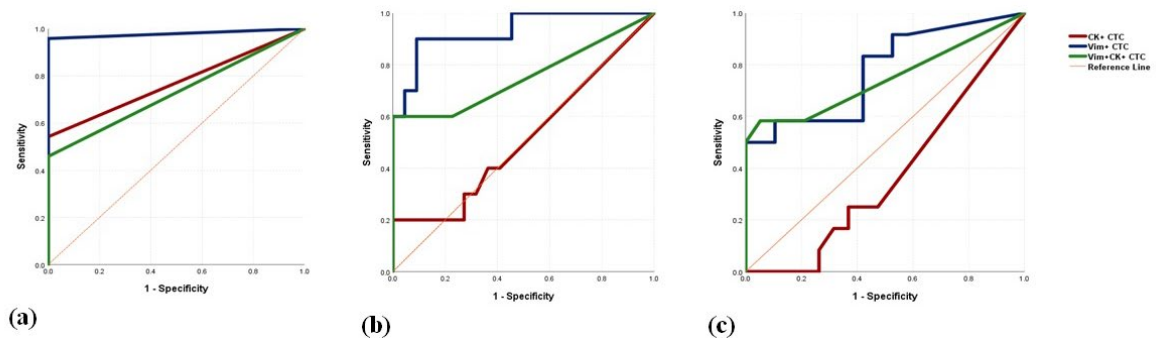
Furthermore, VIM⁺CK⁺CTCs showed substantial accuracy, $73 \pm 0.89\%$, with Using a threshold of 1 cell, TNBC subtype identification showed 46% sensitivity and 100% specificity. In terms of identifying TNBC subtypes, CK⁺CTCs did not demonstrate significant

accuracy, while VIM⁺CTCs exhibited very significant accuracy, $93 \pm 0.049\%$, with With a cut-off of 55 cells, sensitivity was 70% and specificity 96%. Additionally, CK⁺VIM⁺CTCs showed notable differences in accuracy, $75.5 \pm 0.02\%$, with a Demonstrating sensitivity and specificity values of 60% and 100%, respectively, at an 18-cell threshold. When differentiating between various clinical stages of BC, VIM still showed significant accuracy, $78 \pm 0.091\%$, at a threshold of 62 cells, with a sensitivity of 50% and a specificity of 95%. Additionally, the accuracy of VIM+CK+CTCs was significant 75 ± 0.102 , with a sensitivity of 58% and a specificity of 95%. Moreover, the proportion of cells with CK expression was not significant. Table 6 and Fig. 5

Table 6: Diagnostic Tests, FACS Assay, of CK⁺CTCs, Vim⁺CTCs and CK⁺Vim⁺CTCs

Cells/1 ml Blood	Healthy women (n = 10)	BC Women (n = 80)	Cut off	Sensitivity	Specificity	PPV %	NPV %	Accuracy± S.E	P values
CK+CTCs	(100%)	(100%)	1.5	54	100	54	100	77±0.08	0.024*
VIM+CTCs	(100%)	(100%)	1	98	100	96	100	98±0.02	<0.001**
VIM+CK+CTCs	(100%)	(100%)	1	46	100	46	100	73±0.89	0.05*
Cells/1 ml Blood	NTNBC Women (n = 61)	TNBC Women (n = 19)	Cut off	Sensitivity	Specificity	PPV %	NPV %	Accuracy ±S.E	P values
CK+CTCS	(100%)	(100%)	8	20	81	40	36	52±0.116	0.88
VIM+CTCS	(100%)	(100%)	55	70	96	100	7	93±0.049	0.00**
VIM+CK+CTCs	(100%)	(100%)	18	60	100	50	57	75.5±0.02	0.023*
Cells/1 ml Blood	Stage I&II (n = 37)	Stage III (n = 33)	Cut off	Sensitivity	Specificity	PPV %	NPV %	Accuracy ±S.E	P values
CK+CTCs	(100%)	(100%)	5.0	25	63	25	18	37±0.100	0.23
VIM+CTCs	(100%)	(100%)	62	50	95	92	0	78±0.091	0.01**
VIM+CK+CTCs	(100%)	(100%)	6	58	95	58	64	75±0.102	0.02*

** Correlations were Significant at the 0.01 level, * Correlations were Significant at the 0.05 level, PPV: Positive Predictive Value, NPV: Negative Predictive Value

**Figure 5:** Diagnostic Accuracy of (a) CK⁺CTCs, (b) VIM⁺CTCs and (c) VIM⁺CK⁺CTCs for Identifying (a) BC, (b) TNBC and NTNBC and (c) Clinical Stages I-II and III using FACS Assay.

The performance and efficiency of the two assays were evaluated using a recovery curve constructed from known numbers of spiked cells / 1 ml of blood. The recovery of spiked cells was characterized by high linearity for proteins, cytokeratin, and vimentin, with a correlation coefficient $R^2 \geq 0.95$ determined by two assays. The efficiency of the

qRT-PCR assay was >100% because of the low dilution of cells (dynamic range <5-80/100 cells per 1 ml of blood). In addition, ROC curve analysis was employed to determine the accuracy and effectiveness of each assay and its parameters as diagnostic tools. Table 7 and Fig. 6

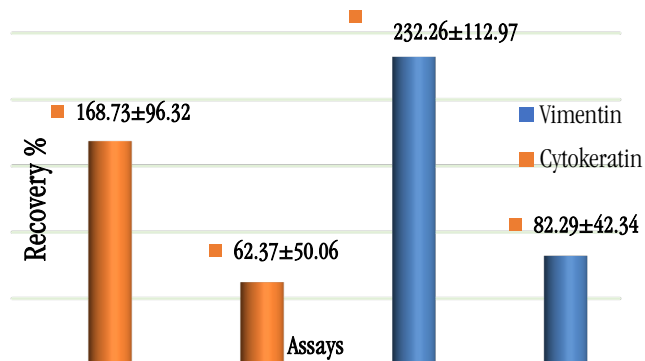
Table 7 (a): The Performance Evaluation for qRT-PCR and FACS Assays

Technique	Cytokeratin		Vimentin	
	qRT-PCR	Flow Cytometry	qRT-PCR	Flow Cytometry
CTCs recovery % as a function of spike-in				
Accuracy	168.73 ± 96.32	62.37 ± 50.06	232.26 ± 112.97	82.29 ± 42.34
Linearity (r^2)	0.98	0.93	0.99	0.91
correlation Coefficient	0.96	0.96	0.99	0.95
LOD (Low Detection limit)	1.57	0.15	2.11	0.25
LOQ (Low Quantitation Limit)	4.75	0.47	6.39	0.76
Precision (RDSr)	0.33	1.40	0.12	1.2
Diagnostic Values of ROC curve to identify BC women from Healthy Women				
Cut-off values	395	1.5	1.7	1
Sensitivity	87	54	100	98
Specificity	83.3	100	100	100
PPV	84	54	92	96
NPV	88	100	100	100
Accuracy ±S.E (AUC)	95±0.039	77±0.08	100±0.0	98±0.02
PValue	<0.001**	0.024*	<0.001**	<0.001**
Diagnostic Values of ROC curve to identify TNBC from NTNBC Women				
Cut-off values	14659	8	1381	55
Sensitivity %	44.4	20	55.6	70
Specificity %	80.0	81	90	96
PPV	90	40	100	100
NPV	20	36	14	7
Accuracy±S.E (AUC) %	74.4±0.102	52±0.116	87.2±0.067	93±0.049
PValue	0.05*	0.88	0.002**	0.001**
Diagnostic Values of ROC curve to identify Stage III from Stage I&II within BC Women				

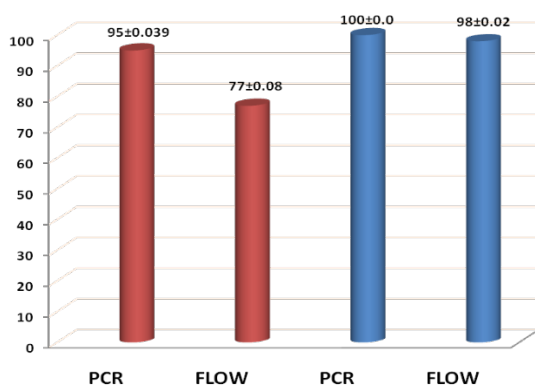
Table 7(b): The Performance Evaluation for qRT–PCR and FACS Assays

Technique	Cytokeratin		Vimentin	
Parameters	qRT–PCR	Flow Cytometry	qRT–PCR	Flow Cytometry
Cut-off Values	13858	5.0	235	6
Sensitivity	30.8	25	77	58
Specificity	68.8	63	81.3	95
PPV	77	25	92	92
NPV	9	18	10	0
Accuracy±S.E (AUC)	53.4±0.111	37±0.100	77.4±0.092	78±0.091
Pvalue	0.759	0.23	0.016*	0.01*

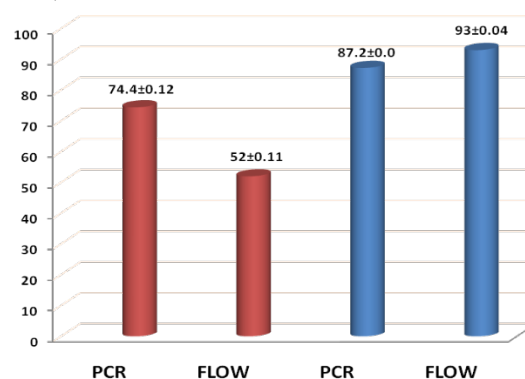
**. The P Value was Significant at the 0.001 level (1-tailed), *. The p Value was Considered to be Significant at the 0.05 Level (1-tailed).



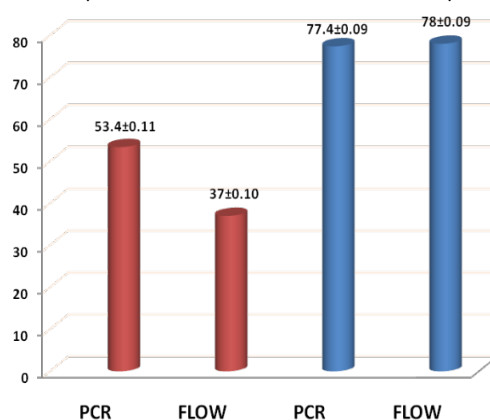
(a) CTCs Recovery Plot as a Function of Spike-in Density for and HCT164 Cells (Positive Control for Cytokeratin) and MDA-MB-231 cells (Positive Control for Vimentin).



(b) Accuracy Plot % by ROC Curve to Identify BC Disease.



(c) Accuracy Plot % by ROC Curve to Identify TNBC.



(d) Accuracy Plot % by ROC Curve to Identify the Clinical Stage of Breast Cancer Disease.

Figure 6: Performance Evaluations for qRT–PCR and Flow Cytometry

Discussion

Breast cancer (BC) is a multifactorial and heterogeneous disease, making accurate classification is essential for accurate diagnosis and prognosis, allowing patients to receive the most effective treatment.

When breast cancer is detected early, treatment can be highly successful and may significantly decrease the mortality rate associated with the disease [21]. Liquid biopsy of “CTCs” is a more suitable and noninvasive method than traditional tissue biopsy for screening and early diagnosis, and provides insight into how cancer develops,

particularly in terms of the metastatic process and Therapeutic response. Variability in methodology, substantial interlaboratory discrepancies in blood sample volumes, differences in sensitivity and specificity evaluations, heterogeneous patient-to-control ratios, and inconsistent data interpretation collectively challenge the assessment of the diagnostic significance of circulating tumor cells (CTCs) in cancer [22]. Hence, the main objective of this study was to develop a technique that is accurate, sensitive, specific, and effective for CTCs detection in blood from BC patients from just one milliliter of blood. Two different assays were used: (1) quantitative real-time RT-PCR, which involves the quantitation of tumor-associated transcripts; and (2) flow cytometry, which is an immunocytochemical method for the detection and quantification. However, CKs and VIM were used in the assessment of BC disease [23]. The results of qRT-PCR demonstrated significantly higher CK-14 levels in BC patients relative to healthy women. Additionally, its expression in TNBC was significantly upregulated compared to that in NTNBC patients. On the other hand, its expression did not significantly change between different clinical stages of BC. Consistent with prior studies, approximately 75% of TNBC cases exhibited positivity for CK-14 and CK5/6, markers closely associated with the basal immunophenotype [24]. CK-14—positive breast cancers are sporadic, originate from glandularly committed progenitor cells, and are characterized as aggressive grade III, steroid hormone receptor—negative tumors, which show an inverse association with HER-2 amplification [25]; i.e., the tumor grade was favorably linked with the expression of CK-14 [23]. CK-14 is also valuable for detecting BC patients with a basal-like phenotype, guiding the use of more aggressive chemotherapy and surgery [24]. CK-19 levels, like those of CK-14, were significantly higher in BC patients than in healthy women; in TNBC, its expression showed no significant variation relative to that in NTNBC patients. In contrast, its expression was significantly lower in the late stage (III) compared to the early stage (I and II) of BC disease. These observations corroborate previous research showing that revealed that CK-19 expression was not detected in healthy individuals *via* real-time RT—PCR [26], and regardless of the histological type, the majority of patients with breast cancer had CK-19 tests that were positive [27], and it was found in more than 95% of breast carcinomas [23]. Importantly, it was reported that CK-19 expression is significantly increased in the peripheral circulation of individuals with early breast cancer, and it has been hypothesized that its expression diminishes as the disease progresses [28]. Using an immunocytochemical method, the number of cells that expressed CKs, CKs+CTCs, was detected in the BCs but not in healthy women. When comparing different clinical stages of BC, the number of CK+CTCs was the highest in early-stage patients, followed by late-stage patients. These results aligned with the qRT-PCR results, although the qRT-PCR assay determines the expression of CKs independent of the number of individual cells, while FACS determines the number of cells that express CKs. On the other hand, the number of CK+CTCs in TNBC was significantly lower than that in NTNBC patients. These findings showed that FACS may be more sensitive than qRT-PCR for identifying TNBC from NTNBC subtype, and this may be due to the combination of CK antibodies (CK-14, 15, 16, and 19). The problem that can contribute to the lack of CKs expression in TNBC is the EMT procedure [29]. Therefore, CKs, including CK-19, are regarded as sensitive markers for identifying early metastasis and malignant prognosis [30]. Vimentin (VIM) is widely recognized as a canonical mesenchymal marker and a key regulator of EMT in various cancers, including breast cancer. As an intermediate filament present in basal-

like and mesenchymal cells, it serves as a specific biomarker for the early detection of BC [31]. These observations are supported by the results of the present study, either qRT—PCR or FACS assays. Regardless of the accuracy, sensitivity, and specificity, both assays demonstrated that VIM levels were significantly elevated in breast cancer patients compared to healthy controls. Furthermore, VIM expression was significantly higher in TNBC patients than in NTNBC cases. Although VIM expression was higher in late-stage (III) BC compared to early-stage (I and II) disease, the difference was not statistically significant. Nonetheless, this highlights the potential to improve the accuracy of BC stage identification using the FACS assay, a combination of CKs and VIM antibodies was used to identify CTCs. The results showed that the percentage of CK+VIM+CTCs was significantly greater in the late stage than in the early stage of BC disease. Our results align with those from earlier research; a study by Seshadri et al. [32] Indicated that VIM expression is significantly negatively associated with progesterone receptor (PR) expression. Another investigation indicated that, firstly, VIM expression was highest in BC patients who were negative for progesterone receptor (PR) and estrogen receptor (ER), and HER2/neu expression. Secondly, while the median VIM expression was considerably lower Among patients with early-stage cancer, it showed considerable variability among individuals; therefore, No statistically significant association was observed between its expression and tumor stage [33]. Moreover, our results align with those of Winter et al., who identified a growing subset of persistent TNBC cells exhibiting elevated levels of VIM. This marker is commonly recognized and serves as a crucial regulator of the EMT process in various cancer types, including breast cancer [31]. Correlation analysis of qRT-PCR revealed a significant positive correlation between basal CK-14 and VIM expression and a negative correlation between CK-19 expression and clinical stage of disease. Various mechanisms have been proposed for the loss of CK-19 expression, including dysregulated expression and increased proteolytic degradation. Moreover, EMT has been linked to the growth of cancer, as epithelial-derived tumor cells undergo a change in their phenotype to a more basic mesenchymal phenotype [34]. Correlation analysis of FACS results showed strong positive correlations between clinical disease stages and cells expressing VIM (VIM+CTCs) or cells expressing CKs and VIM (CK+VIM+CTCs). Due to incomplete EMT, CTCs may develop a variety of EMT phenotypes and exhibit unique characteristics that contribute to their plasticity. Because they are more adaptable and can change. In response to environmental cues, tumor cells may adopt epithelial or mesenchymal states; it is hypothesized that cells in a partial EMT state exhibit poorer outcome than those undergoing complete EMT. These findings suggest that CTCs undergoing EMT may be connected to full or partial CK-19 substitution by VIM [35, 36]. The diagnostic test, the qRT-PCR assay, showed that VIM expression, followed by CK-19 expression, had the highest accuracy, sensitivity, and specificity for identifying BC, particularly the TNBC subtype, or distinguishing between the clinical stages of BC. The FACS assay demonstrated that VIM, followed by CKs, remains the most effective diagnostic test exhibiting the highest accuracy, sensitivity, and specificity for identifying BC, particularly the TNBC subtype. However, these methods had low specificity for identifying clinical stages of disease. These results are consistent with a previous study demonstrating that VIM plays a critical role in the early and accurate diagnosis of breast cancer, specificity, and sensitivity [37]. The assay performance and efficiency values for two assays, qRT-PCR and FACS, demonstrated that (1) qRT-PCR was more sensitive than flow cytometry as a diagnostic test for CKs and/or VIM,

but flow cytometry was more specific for identifying BC disease; and (2) to distinguish between TNBC and NTNBC subtypes or between clinical stages of BC disease, VIM was more specific and sensitive, followed by CKs, which were detected using two assays.

Conclusions

The current study concluded that a combination of CKs and VIM may be more advantageous for analyzing and identifying phenotypes in breast cancer patients, owing to tumor heterogeneity and variability associated with breast carcinoma, as evidenced by the distinct staining patterns of individual antibodies. Furthermore, to enhance both sensitivity and specificity, it is advisable to employ both techniques for the EMT differentiation phenotypes and other characteristics of breast cancer, thereby facilitating early identification and contributing to effective therapeutic strategies.

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Declarations

Ethics Approval and Consent to Participate

A signed informed consent was obtained from each participant in the present study in accordance to the rules of Ethics Committee, Medical Research Institute, Alexandria University.

Consent for Publication

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