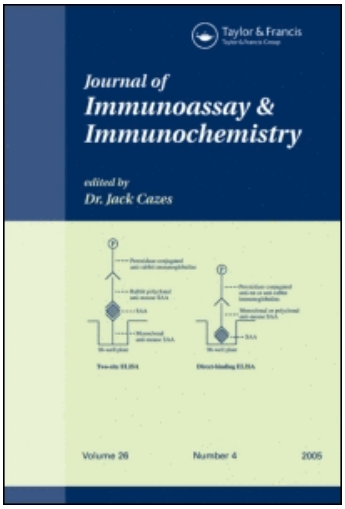

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Utility of Slot-Blot-ELISA as a New, Fast, and Sensitive Immunoassay for Detection of Carcinoembryonic Antigen in the Urine Samples of Patients with Various Gastrointestinal Malignancies

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Abstract: Carcinoembryonic antigen (CEA) is the most widely used clinical tumor marker. CEA immunoassay has found acceptance as a diagnostic adjunct in clinical diagnosis of gastrointestinal tumors (GIT). Several immunoassays have been established for detection of CEA in plasma, serum, tissue, feces, and urine of cancer patients using polyclonal or monoclonal antibodies raised against CEA. Some of these assays display both high sensitivity and specificity for the detection of CEA. However, these assays require special and highly expensive equipment and the procedures require long periods for their completion. In the present study, we established a Slot-Blot Enzyme Linked Immunosorbent Assay (SB-ELISA), based on anti-CEA monoclonal antibody (CEA-mAb), as a new, simple, fast, cheap, and non-invasive

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immunodiagnostic technique for detection of CEA in the urine of GIT patients. Urine and serum samples were collected from 248 GIT patients (58 with pancreatic cancer, 20 with hepatoma, 23 with ampullary carcinoma, 15 with hilar cholangiocarcinoma, 28 with gastric cancer, 14 with esophageal cancer, and 90 with colorectal cancer). Moreover, urine and serum samples were collected from 50 healthy individuals to serve as negative controls. The traditional ELISA technique was used for determination of CEA in the sera of GIT patients using anti-CEA monoclonal antibody. A comparison between the results of both techniques (ELISA and SB-ELISA) was carried out.

The traditional ELISA detected CEA in the sera of 154 out of 248 GIT patients with a sensitivity of 59.8%, 51.7% positive predictive value (PPV) and 75.37% negative predictive value (NPV). In addition, it identified 15 false positive cases out of 50 healthy individuals with a specificity of 70%. The urinary CEA was identified by a Western blotting technique and CEA-mAb at a molecular mass of 180 Kda. The developed SB-ELISA showed higher sensitivity, specificity, PPV, and NPV (70.1%, 78%, 62.4%, and 82.13%, respectively) for detection of CEA in the urine of GIT patients. The semi-quantitative SB-ELISA showed a higher overall efficiency of 72.8% versus 63.4% in the case of the quantitative ELISA, for detection of CEA.

In conclusion, SB-ELISA is more efficient for detection of CEA in gastrointestinal tumors. It is a simple, rapid, non-invasive, and sensitive assay. Moreover, all steps of the SB-ELISA are performed at room temperature, without the use of expensive equipment; this may enhance the application of this assay in field studies and mass screening programs.

Keywords: Carcinoembryonic antigen, Gastrointestinal tumors, Western blotting, SB-ELISA

INTRODUCTION

Cancer is a universal problem; it is the leading cause of death in many different parts of the world. Cancer diagnosis with serological testing is still one of the major research priorities among clinical biochemists.[1]

Carcinoembryonic antigen (CEA) is the most widely used tumor marker for monitoring colorectal cancer patients.[2] CEA was first identified, in 1965, by Gold and Freedman as a tumor marker-specific antigen which is present in extracts of human colon cancer tissue and in normal fetal gastrointestinal tract epithelial cells.[3,4] CEA comprises a large family of cell-surface glycoproteins with common antigenic determinants residing in the protein portion of the molecule. It is composed of a single polypeptide chain consisting of 30 amino acids and has a molecular mass between 160 and 300 Kda, with 45% to 57% of the molecule consisting of carbohydrate.[5,6]

CEA immunoassays using monoclonal antibodies against CEA, derived from a colonic tumor, have found acceptance as diagnostic and prognostic tools for colorectal cancer patients[7–14] and monitoring of patients following colorectal tumor resection.[15–20] Several immunoassays have been established for the detection of CEA in plasma,[21] serum,[22] tissues,[23] feces,[21] and urine of cancer patients,[24–26] using polyclonal or monoclonal antibodies against CEA. Some of these assays display both high sensitivity and specificity for the

detection of CEA. However, they require special and highly expensive equipment and the procedures require long periods for their completion. The two most commonly used methods for CEA analysis are enzyme immunoassay[7,27] and radioimmunoassay.[9,28–30] Both of them are commercially available.

In the present study, we aimed to establish a Slot-Blot Enzyme Linked Immuonsorbent Assay (SB-ELISA) as a new, rapid, inexpensive, and simple diagnostic technique for the detection of CEA secreted into the urine of GIT cancer patients, using CEA monoclonal antibody (CEA-mAb). The results of SB-ELISA will be compared with a traditional ELISA technique for the detection of CEA in the sera of the same patients.

EXPERIMENTAL

Urine and Blood Samples

Urine and blood samples were obtained from 248 GIT patients (hospitalized in the Gastroenterology Center, Mansoura University, Egypt). These patients were: 20 with hepatoma (12 M & 8 F with mean age of 56.65 $\pm$ 6.85 y); 23 with ampullary carcinoma (11 M & 12 F with mean age of 55.83 $\pm$ 7.67 y); 15 with hilar cholangiocarcinoma (9 M & 6 F with mean age of 51.67 $\pm$ 8.18 y); 58 with pancreatic cancer (38 M & 20 F with mean age of 54.33 $\pm$ 8.6 y); 28 with stomach cancer (17 M & 11 F with mean age of 53.04 $\pm$ 11.01 y); 14 with esophageal cancer (9 M & 5 F with mean age of 57.79 $\pm$ 12.01 y); and 90 colorectal cancer patients (55 males and 35 females with mean age of 50.8 $\pm$ 11.36 y). They were diagnosed in accordance with clinical and pathological examinations. The study protocol respected the most recent Declaration of Helsinki (Edinburgh, 2000), and all of the patients gave consent to the use of their samples and clinical data for research purposes after being informed about the nature of the study. In addition, 50 urine and blood samples were collected from healthy individuals (38 M & 12 F with mean age 52 $\pm$ 8.1 y) as a negative control group. Controls were neither alcohol drinkers nor tobacco users. All urine and serum samples were stored at -20ºC until used.

CEA Extract Preparation

CEA extract was prepared from colon cancer tissue by the method described by Newman et al.[31] using 1.2 M perchloric acid. The protein content of the CEA extract was measured by the method of Lowry et al.[30] and then stored at -70ºC until used.

Monoclonal Antibody

An IgG mouse monoclonal antibody (designed CEA-mAb) developed against CEA using a hybridoma technique, was used in the present study. The anti-CEA-mAb was produced at the Biotechnology Research

Center (BRC), New Damietta City, Egypt, and was kindly provided by Prof. Dr. Abdelfattah M. Attallah, Director of the BRC.

Western Blotting

SDS-PAGE was carried out in 0.75 mm thick, 7.5% or 10% vertical slab gels (Bio-Rad) under reducing conditions according to the method of Laemmli.[33] After separation of urine polypeptides and CEA extract on SDS-PAGE, the separated proteins were electrotransferred onto a nitrocellulose filter (NC) (0.45 μm pore size, Bio-Rad, USA) in a Bio-Rad protein transfer unit, as described by Towbin et al.[34] The blotting was carried out with a constant 60 volts for two hours. The NC filter was blocked using 5% (w/v) non-fat dry milk dissolved in 0.05 M Tris-HCl buffer containing 0.15 M NaCl (TBS), pH 7.4. The NC filter was then rinsed in TBS and incubated with anti-CEA monoclonal antibody, diluted in blocking buffer (1:200), with constant shaking. The blots were washed three times (30 min each) in TBS, followed by two hours incubation with 1:500 diluted Goat anti-mouse alkaline phosphatase conjugate (Sigma) in TBS. After washing three times with TBS (15 min each), the blots were soaked in alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyl phosphate [BCIP], nitro blue tetrazolium [NBT] and 0.1 M Tris buffer, pH 9.6 (Sigma). The color reaction was observed within five minutes, then the reaction was stopped by distilled water and the filter was dried and kept in the dark.

Purification of CEA from Preparative Slab Gel Using Electroelution

SDS-PAGE adapted for preparative purposes is prepared by increasing the thickness.[35] Thirty ug/gel of CEA extract were applied to the gel. electrophoresis using a constant voltage. Electroelution was carried out using Electroeluter unit (Hybaid) at constant voltage of 200 volts for three hrs.[36] Electroeluted antigen was dialyzed in a dialysis sack (Sigma) with a cutoff of 12–14 Kda against one liter of phosphate-buffered saline (Sigma), pH, 7.2 overnight at +4ºC with constant stirring. After dialysis, the electroeluted antigen was concentrated using 10 gm polyethylene glycol for one hr at room temperature and then reconstituted in PBS, pH 7.2. The protein content of the antigenic solution was measured colorimetrically using the method of Lowry et al.[32] and stored at −20ºC until used.

Indirect ELISA

The method described by El-Masry et al.[37] was followed. Briefly, a polystyrene flat-bottom microtiter plate was coated with 50 μL of diluted serum (1:50) in carbonate-bicarbonate buffer (pH 9.6). The coated plate was incubated overnight at room temperature. After blocking with 0.2% non-fat milk in coating buffer, 50 μL (per well) of 1:200 diluted anti-CEA mAb in

0.05 (v/v) phosphate buffered saline (PBS)-Tween (PBS-T20) was added and the plate was incubated at 37ºC for two hours. After washing, 50 μL (per well) of 1:500 anti-mouse IgG whole molecule alkaline phosphatase conjugate (Sigma) diluted in 0.2% (v/v) non-fat milk in PBS-T20, were added and the plate was incubated at 37°C for one hour. One milligram of *para*-nitrophenyl phosphate (Sigma) per mL was added as a substrate solution and the absorbance was read at 405 nm using an EL311 microplate autoreader (Bio-Tek instruments). Cut-off level of ELISA above or below which the tested samples were considered positive or negative was calculated as the mean concentrations of 50 serum samples from healthy individuals +2SD.

Slot-Blot Enzyme-Linked Immunosorbent Assay (SB-ELISA)

The SB-ELISA of El-Masry et al.[37,38] was used with some modifications to detect CEA secreted in urine of colorectal cancer patients. In brief, The NC filter (Sigma) was washed in a bath of distilled water followed by soaking in a bath of PBS (pH 7.4) for two minutes. The NC filter was overlaid on a filter paper which had been presoked in PBS and held in the Manifold Slot Blot apparatus (Sigma). Fifty microliters of urine samples of cancer patients and healthy individuals were added to each well of the Slot-Blot apparatus; the purified CEA was used as a positive control and bovine serum albumin (BSA, Sigma) was used as a negative control. After suction, the NC filter was air dried and then washed in 0.3% PBS-T20 for one minute with shaking. Blocking of the non-specific binding sites on the filter was done with 2% (w/v) non-fat dry milk in PBS-T20 for five minutes on a shaker. After removal of the blocking solution, the anti-CEA-mAb was added in a dilution of 1:500 in PBS-T20 for five minutes with continuous shaking. After washing with PBS, anti-mouse IgG alkaline phosphatase conjugate (Sigma) was added at a dilution of 1:500 in blocking buffer for five minutes with continuous shaking. The filter was washed with PBS and the color was developed after addition of alkaline phosphatase substrate solution (BCIP/NBT). The reaction was stopped with distilled water and the results were recorded.

In each run, a positive control and negative controls were included. SB-ELISA allows a semi-quantitative reading of CEA according to the intensity of the color produced. The purple color varied in the intensity from weak positive (1+, 2+) to strong positive (3+, 4+). A colorless slot was produced in case of a pure negative test result. The resulting color of the tested samples was then visually compared to the color of the positive and negative controls.

RESULTS

Identification of CEA in the Cancerous Tissue Extract

The carcinoembryonic antigen was detected and identified in the colon cancer tissue extract using a Western blotting technique and CEA-mAb. The result

showed that an intense, sharp and single immuoreactive band was observed at a molecular mass of 180 Kda in the colon cancer tissue extract, as shown in Figure 1.

CEA Purification

CEA was isolated and purified from human colon cancer tissue extract using electroelution from 7.5% polyacrylamide preparative slab gels. The immunoreactivity of the purified antigen was tested against mouse CEA-mAb by the Western blotting technique and alkaline phosphatase immunostaining. An intense, sharp band was detected at a molecular mass of 180 Kda, as shown in Figure 2.

Identification of Urinary CEA Using Anti-CEA mAb

Carcinoembryonic antigen secreted in the urine of cancer patients was detected and identified using CEA-mAb by the Western blotting technique. The urine samples from cancer patients and healthy individuals were resolved onto 7.5% SDS-PAGE. Then, the separated polypeptides were electrotranfered onto an NC filter and the immunoreaction was performed using CEA-mAb and alkaline phosphatase immunostaining. An intense band was observed at 180 Kda in the urine of a cancer patient (lane P), and no reaction was observed in urine from a healthy individual (lane N) as shown in Figure 3.

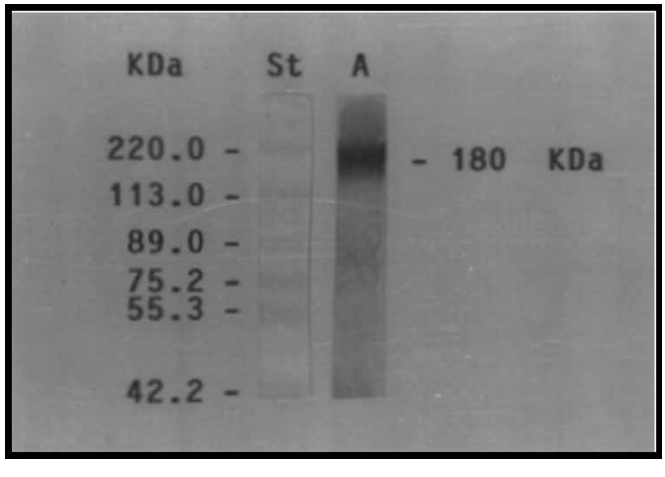

Figure 1. Western blot analysis of the perchloric acid human colon tumor tissue extract (lane A), resolved onto 7.5% SDS-PAGE, using CEA-mAb as a primary antibody and anti-mouse IgG alkaline phosphatase conjugate as a secondary antibody. The immunoreaction was visualized with NBT/BCIP substrate solution. St: prestained molecular weight markers.

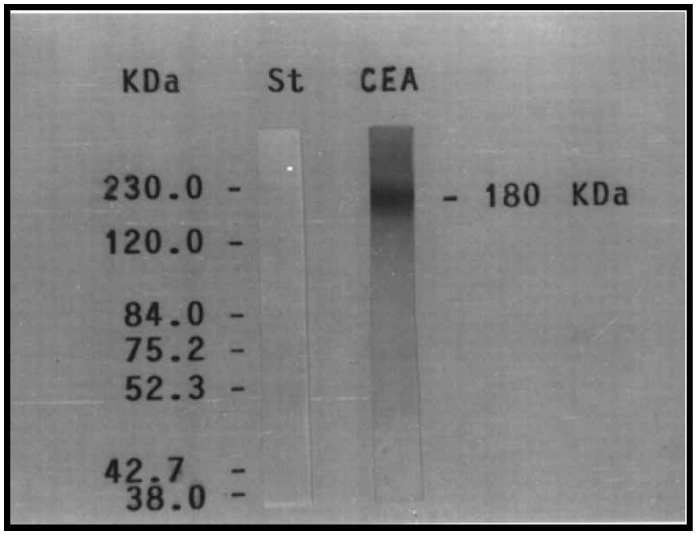

Figure 2. Immunoblots of CEA-mAb target antigen in a purified CEA, resolved onto 7.5% SDS-PAGE using mouse CEA-mAb and alkaline phosphatase immunostaining. St: prestained molecular weight markers.

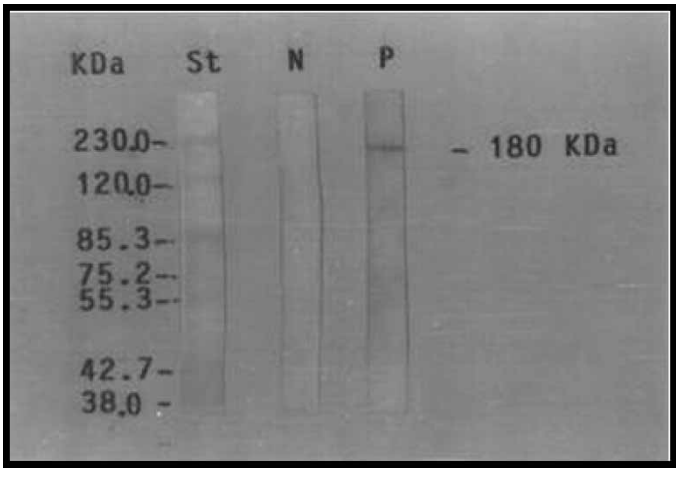

Figure 3. Western blot of urine samples from colon cancer patient (lane P) and healthy individual (lane N). Urine samples were separated on 7.5% acrylamide gel in presence of SDS. The resolved proteins were electroblotted onto nitrocellulose membrane and probed with CEA-mAb. Binding of CEA-mAb was detected using goat anti-mouse IgG alkaline phosphatase conjugate and NBT/BCIP substrate system. St: prestained molecular weight markers.

SB-ELISA for Detection of Urinary CEA

In serial dilution of CEA, SB-ELISA was able to detect 0.49 μg/mL as the lowest detectable CEA concentration (Figure 4). Urine samples from 248 GIT patients and 50 healthy individuals were subjected to SB-ELISA for detection of urinary CEA using CEA-mAb. The result showed different degrees of positivity to CEA according to the intensity of the blot color compared with white background of the negative control (BSA), as shown in Figure 5. The SB-ELISA results were classified, according to the visual reading of the colored blots, into (++++), (+++), (++), (+), and negative, according to the concentration of CEA in the urine samples as shown in Table 1. The SB-ELISA data showed that the assay detected 178 out of 248 GIT patients with sensitivity of 70.1% and 62.4% positive predictive value. In the case of healthy individuals, the assay detected 11 false positive cases from 50 healthy individuals; this revealed a 78% specificity and 82.13% negative predictive value. The overall efficiency of SB-ELISA for the detection of CEA was 72.8% (Tables 2 and 3).

Indirect ELISA Results for Detection of Serum CEA

Serum samples from 248 GIT patients and 50 healthy individuals were tested with indirect ELISA for detection of CEA in blood samples using CEA-mAb.

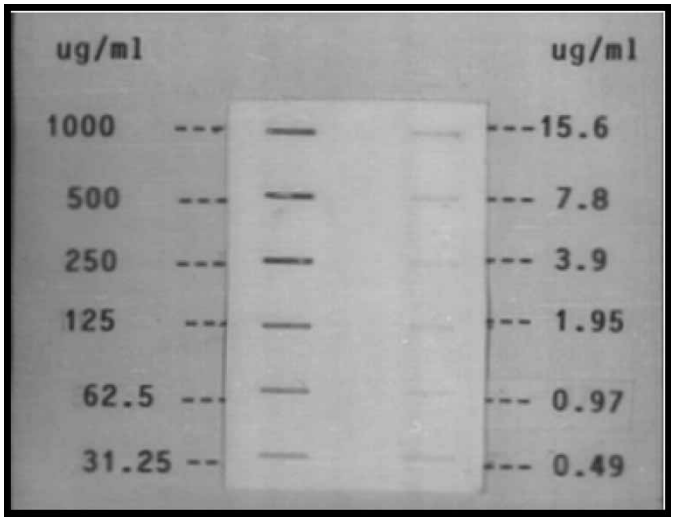

Figure 4. SB-ELISA analysis for serial concentrations of purified CEA to determine the least detection limit using CEA-mAb and alkaline phosphatase immunostaining. It was observed that SB-ELISA was able to detect up to 0.49 μg/mL.

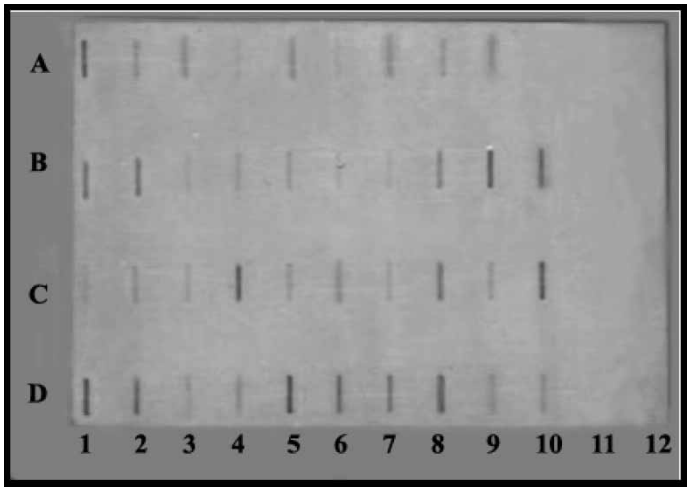

Figure 5. SB-ELISA analysis for urine samples of GIT patients using CEA-mAb and alkaline phosphatase immunostaining. The assay allows a semi-quantitative reading; i.e., faint slots (1+ or 2+ color intensity) are considered weak positive and dark slots (3+ or 4+ color intensity) are considered strong positive. Wells B11, B12, C11, C12, D11, and D12 represent urine samples from healthy individuals, wells A10, A11, and A12 represent a non-specific protein (Bovine Serum Albumin) as negative control and wells D1 and D2 represent a purified CEA as a positive control.

Table 1. SB-ELISA Results for detection of CEA in the urine samples of different GIT patients and healthy individuals groups

		SB-ELISA results				
Status	No	−Ve	+	++	+++	++++
Pancreatic cancer	58	14	0	15	22	7
Hepatoma	20	6	2	5	6	1
Ampullary carcinoma	23	10	1	5	6	1
H. Cholangiocarcinoma	15	3	0	3	3	6
Stomach cancer	28	11	1	2	9	5
Esophageal cancer	14	5	3	5	0	1
Colorectal cancer	90	21	8	17	34	10
Total GIT patients	248	70	15	52	80	31
Healthy individuals						
With urinary tract infection	20	15	3	2	0	0
Without urinary tract infection	30	24	5	1	0	0
Total healthy individuals	50	39	8	3	0	0

Table 2. Results of SB-ELISA and ELISA techniques

		SB-ELISA results		ELISA results	
Status	No	+Ve	−Ve	+Ve	−Ve
Pancreatic cancer	58	44	14	40	18
Hepatoma	20	14	6	13	7
Ampullary carcinoma	23	13	10	11	12
H. Cholangiocarcinoma	15	12	3	8	7
Stomach cancer	28	17	11	16	12
Esophageal cancer	14	9	5	8	6
Colorectal cancer	90	69	21	58	32
Total GIT patients	248	178	70	154	94
Healthy individuals: With urinary tract infection	20	5	15	7	13
Without urinary tract infection	30	6	24	8	22
Total healthy individuals	50	11	39	15	35

The results showed that ELISA was able to detect the CEA in 154 out of 248 GIT patients with a sensitivity of 59.8% and 51.7% positive predictive value. In addition, the assay had false positive results for 15 out of 50 healthy individuals with a specificity of 70% and 52.2% negative predictive value. The overall efficiency of the indirect ELISA for detection of CEA was 63.4% (Tables 2 and 3).

DISCUSSION

The carcinoembryonic antigen molecule is an oncodevelopmental human tumor marker, which is a surface glycoprotein that may be released into the interstitial space and, hence, into the circulation of the tumor bearing patient, where it can be detected by immunoassay.[28] Moreover, CEA and other antigens with a comparable molecular weight can be detected in the urine of healthy and diseased persons.[39–42]

Several immunodiagnostic assays based on CEA-mAb for the detection of CEA in different body fluids of GIT patients have been described.[7,9,11,12,17,26,27,29] These assays cannot be easily applied in the field, because they need a long time for their completion and special and very expensive equipment. Therefore, it is very important that a rapid, simple and reliable test for detection of CEA be developed.

In the present study, the SB-ELISA developed based on anti-CEA-mAb for the detection of CEA excreted in the urine of GIT patients, is simple, rapid, sensitive, and specific enzyme immunoassay. The assay could, therefore, be used in the field as part of a mass screening program. The urine sample was used neat, i.e., without any pretreatment; the assay needs no sophisticated

Table 3. Evaluation of SB-ELISA compared with ELISA for detection of CEA in colorectal cancer patients

		SB-ELISA			ELISA		
Status	No	Sen. (%)	PPV (%)	NPV (%)	Sen. (%)	PPV (%)	NPV (%)
Pancreatic cancer	58	75.8	80.0	73.6	70.0	72.7	66.0
Hepatoma	20	70.0	56.0	86.7	65.0	46.6	83.0
Ampullary carcinoma	23	56.5	54.0	79.6	47.8	42.0	74.5
H. Cholangiocarcinoma	15	80.0	52.0	92.8	53.0	34.8	83.0
Stomach cancer	28	60.7	60.7	78.0	57.0	51.6	74.5
Esophageal cancer	14	64.0	45.0	88.6	57.0	53.0	85.0
Colorectal cancer	90	76.7	86.3	65.0	64.4	79.5	52.2

Sen. (%): Sensitivity; PPV (%): Positive predictive value; NPV (%): Negative predictive value.

equipment and 96 urine samples could be run in about 30 min. In addition, all steps were done at room temperature.

We evaluated the sensitivity and specificity of SB-ELISA for the detection of CEA excreted in urine of GIT patients in comparison with the traditional ELISA. We found that SB-ELISA had a higher sensitivity and specificity (70.1% and 78%, respectively) than the detection of CEA in serum samples of colorectal cancer patients by ELISA, which showed 59.8% sensitivity, and 70% specificity. The target antigen of CEA-mAb was identified at 180 Kda in urine of colon cancer patient by the Western blotting technique, as well as in the colon cancer tissue extract.

In conclusion, SB-ELISA has a number of advantages that make it a preferable technique over other diagnostic assays; it is simple, rapid, non-invasive, specific, and sensitive for the detection of CEA. This will enhance the application of this assay in mass screening and control programs of gastrointestinal tumors.

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