

ENZYME IMMUNOASSAY TO DETECT CARCINOEMBRYONIC ANTIGEN IN HUMAN FECAL FECES

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SUMMARY

A solid-phase enzyme immunoassay (sandwich) using both, monoclonal and polyclonal antibodies against Carcinoembryonic Antigen was developed. The assay has as lowest limit of detection, 3 µg/g of CEA in fecal feces and has 3 incubation steps which can be performed in one day. Polystyrene plates coated with a monoclonal antibody against CEA are first incubated with fecal feces extract samples. Bound CEA is detected by addition of polyclonal antibody raised in sheep labelled with alkaline phosphatase. The developed colour is proportional to the amount of CEA present in the samples. Some features of the system like intra and inter assay variation, (10%CV), dilution studies, parallelism test, recovery percent (105.7%), are reported. Also the results obtained with 18 pathological samples (mean value of CEA: 144.26 µg/g) and 28 control samples (mean value: 69.9 µg/g) assayed using the described ELISA system are shown.

RESUMEN

Se describe el montaje de un ensayo inmunoenzimático tipo sandwich usando un anticuerpo monoclonal y anticuerpos policlonales contra CEA. El ensayo detecta 3 µg/g de CEA como valor mínimo detectable en heces fecales y tiene tres pasos de incubación que pueden ser realizados en un día. La placa de poliestireno recubierta con el anticuerpo monoclonal anti-CEA, se incubó primeramente con extracto de heces fecales. El CEA unido se detecta por la unión de los anticuerpos policlonales obtenidos en carnero, marcados con fosfatasa alcalina. El color desarrollado es proporcional a la cantidad de CEA presente en las muestras. Se reportan algunas características del sistema, como la variación intra e inter ensayo, (CV%), estudios de dilución, prueba de paralelismo y porcentaje de recobrado, (105.7%). También se muestran los resultados obtenidos al probar muestras de heces fecales de 18 pacientes con cáncer de colon (valor de CEA promedio: 144.26 µg/g) y 28 muestras controles de individuos sanos (valor promedio: 69.9 µg/g) por el sistema aquí descrito.

INTRODUCTION

The Carcinoembryonic Antigen (CEA), first described by Gold and Freedman in 1965 is a glycoprotein of approximately 200 000 molecular weight. The CEA belongs to a family of related glycoproteins, that show strong cross-reaction among them. Many immunological techniques have been setup for the detection and quantification of CEA, mainly in human serum, using radioisotopic and ELISA methods. The effectiveness and usefulness of these assays are well proven in the

prognosis and follow-up of cancer patients, specifically in colorectal neoplasms. The early diagnosis of malignancies are biased due to the frequently high level of CEA in non-neoplastic diseases, and the low amount of the antigen in the blood stream.

Moreover, the observation of Sugarbaker in 1976 and Fujimoto *et al.* in 1978, suggest that CEA is released more into the gut lumen than into the blood stream.

The purpose of this work is to design a system able to measure CEA in fecal feces and to obtain a more realistic approach of the early detection of colon cancer; using biological fluids other than serum, (Tatsuta *et al.*, 1980; Tatsuta *et al.*, 1982; Castelli *et al.*, 1986).

MATERIALS AND METHODS

Monoclonal Anti-CEA Antibodies

Hybridoma secreting monoclonal anti CEA antibody was obtained according to Tormo *et al.* in 1989. Also in this article the very low cross-reactivity of the monoclonal antibody with normal cross-reacting antigen is reported.

The ascitis fluid obtained was purified using Protein A Sepharose chromatography (Pharmacia-LKB, Uppsala, Sweden).

Antigen

CEA was purified from liver metastases of primary colon adenocarcinoma, as described by Hammarstrom *et al.*, in 1976, with some modifications. Briefly, the acid soluble fraction was purified by affinity chromatography using a Sepharose 4B BrCN activated column, coupled with anti-CEA monoclonal antibody, according to manufacturer's procedure. (Pharmacia-LKB, Uppsala, Sweden).

The column was equilibrated with Phosphate Buffer Saline, (PBS) 0.15 mol/L, pH 7.2. The specific antigen peak was eluted using glycine buffer 0.2 mol/L pH 2.8, and then, extensively dialyzed against PBS. Its concentration was determined by an ELISA method for CEA (Velandia *et al.*, report in press). The purity level was evaluated by SDS-PAGE, (polyacrilamide gel electrophoresis with sodium dodecyl sulfate), using 7.5% gels, (Laemmli, 1970).

Polyclonal Antisera

Sheeps were immunized with purified antigen, with one intramuscular injection of about 100 g of purified CEA in Freund's complete adjuvant for the first immunization, (Sigma, St. Louis, USA). For the remain immunizations was used Freund's incomplete adjuvant, every week during one month. The booster was applied after fifteen days. The monospecificity was achieved by extensive adsorption against extracts from different normal organs i.e.: colon, lung, spleen, stomach and liver and human serum. The extracts were prepared in a proportion 3:1 (g/mL) of the tissue and distilled water followed homogenization in Polytron at 10 000 rpm, 10 min. For the human serum, the extraction step was omitted.

The immunoadsorbents were done by polymerization of the extracts and the human pool serum, with glutaraldehyde, as described by Avrameas and Ternynck in 1969; and Velandia *et al.*, report in press. Ouchterlony and Nielssohn method (1978), was used to assess the antisera titer and its specificity.

The antibodies were purified by affinity chromatography in column of AH-Sepharose 4B coupled to crude metastases extract containing CEA, according manufacturer's procedure (Pharmacia-LKB, Uppsala, Sweden). The concentration was determined by Folin-Phenol method described by Lowry *et al.*, in 1951.

Antibody Labelling

The purified sheep anti-CEA antibodies were labelled with alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany) in accordance with Avrameas, 1968.

Standard and Control Preparation

Different fecal feces samples from "normal" individuals were extracted to a proportion 1:10 (g/mL) with extraction buffer Tris 0.015 mol/L, NaCl 0.15 mol/L, penicillin 500 U/mL, streptomycin 0.5 mg/mL, phenyl methyl sulfonyl fluoride (PMSF) (Sigma, St. Louis, USA) 1 mmol/L, pH 8.0. The extracts were homogenized until total dissolution and ultracentrifuged at 30 000 rpm, at 4°C during 60 min. The pellet was discarded and the collected supernatant was filtered by 0.2 µm filter (Sartorius, Göttingen, Germany). The extracts were frozen at -20°C until use.

These samples were tested by the method previously reported by Velandia *et al.*, (report in press). The specimens with the highest concentration were pooled to obtain a standard with final concentration of 1000 ng/mL. The adjustment of the CEA levels was achieved with proper dilution with negative fecal feces extract for CEA.

The calibration curve had five points, 1000 ng/mL, 500 ng/mL, 250 ng/mL, 125 ng/mL, 62.5 ng/mL, obtained by double serial dilutions of the highest concentration point.

The control was prepared with pooled extracts used for the standard, properly diluted with negative fecal feces extract, to achieve a concentration of 160 ng/mL.

The dilution buffer for samples, standard and control was PBS 0.15 mol/L, 0.05% Tween 20 and sheep serum at 5%.

Enzyme Immunoassay

Polystyrene plates (High binding, Costar, Cambridge, USA), were coated with 50 µL per well of monoclonal anti CEA antibody diluted with coating buffer sodium carbonate/bicarbonate 0.1 mol/L, pH 9.6, to a concentration of 15 µg/mL.

The plates were incubated overnight at 4°C, and then washed with washing buffer, PBS 0.15 mol/L, and 0.05% of Tween 20.

The standard curve, control and samples (diluted 1:160 with dilution buffer from extracts), were added to the plate. The calibrators as well as the samples were assayed in duplicates of 50 µL per well each one.

The plates were incubated in humidity chamber at 37°C during one hour, then were washed as described above and the conjugate diluted 1:400 in washing buffer was added (50 µL per well).

The plates were incubated at 37°C during one hour and washed. The enzymatic activity bound to the plate was revealed by adding 5 mg of p-nitrophenyl phosphate diluted in 5 mL diethanolamine buffer 1 mol/L, 1 mmol/L Mg₂Cl pH 9.8, 50 µL per well. The reaction was stopped after 30 min, at room temperature by the addition of 50 µL per well of 3 mol/L NaOH. The absorbance values at 405 nm were read in a plate reader Multiskan Titertek, (High Wycombe, England).

Intra and Inter Assay Variation

Variation was assessed measuring pools of fecal feces extract in which Carcinoembryonic Antigen was added to a concentration range that would fall within the linear segment defined by the standard points, and were assayed in nine replicas per plate during 7 days. The coefficient of variation (CV%) was determined to give a statistical estimation of the variation of mean of replicates (Snedecor and Cochran, 1980).

Lowest Detectable Concentration

It was determined according to Llano *et al.*, in 1989.

Dilution Studies

Ten fresh fecal feces samples extracted following the extraction method for samples (see below), were diluted in an extensive dilution range, to obtain overlapping zones with the standard curve. The analytical dose-response curves of each sample and the standard curve were linearized following the method suggested by Smith *et al.*, 1987. The linearity was assessed by linear regression, and the coefficients of correlation of each line were calculated. The parallelism was checked by X² method as described by Acevedo *et al.*, 1980.

Recovery

Different fecal feces samples extracts were quantified and a fixed amount of CEA was added and the samples were re-estimated. The CEA recovered was calculated for each specimen by subtracting the endogenous CEA concentration from the CEA present in the enriched sample.

Control Subjects and Specimens

Eighteen samples of colorectal cancer patients ranging in age from 49 to 90 years, with clinical and histological confirmed diagnosis were studied. The samples were extracted in a proportion 1:10 (g/mL) in extraction buffer and centrifuged at 14000 rpm, during 25 min, at 4°C. The pellets were discarded and the collected supernatants were stored deep frozen (-20 °C).

Twenty eight control samples from normal population ranging in ages from 50 to 83 years, underwent the same treatment. The extracts were diluted 1:160 in dilution buffer with 5% sheep serum, and sampled on the plates in duplicates. The Mann-Whitney test, (Mann and Whitney, 1947) was applied in order to estimate significant differences between the pathologic group and the control group.

RESULTS

Monoclonal anti-CEA antibodies.

A typical elution profile for the monoclonal antibody using Protein A Sepharose chromatography was obtained.

The SDS-PAGE showed a single band corresponding to the purified antibody, in a zone of molecular weight of 150 000 Daltons.

Antigen.

A single band of an apparent molecular weight of 180 000 Daltons was obtained when the antigen was purified and analyzed by SDS-PAGE.

Polyclonal Antisera.

There was no reaction between the adsorbed antisera and different normal organ extracts and human serum, so, the monospecificity was achieved.

Enzyme Immunoassay.

The optimal signal corresponding to 1000 ng/mL of standard concentration was obtained through a cross-titration of the reactants, finding the best suitable condition for the coating monoclonal antibody: 15 g/mL and for the conjugate with alkaline phosphatase: 1:400.

The curve obtained exhibits a 45° angle respect to the abscissa axis, (Fig. 1).

Intra and Inter Assay Variation

Results are given in tables 1 and 2.

For the intra assay variation 9 replicas per pool were analyzed. The mean, standard deviation and CV(%) are reported. The coefficient of variation was 5.68%.

For inter assay variation the same sample pools were analyzed during seven consecutives days. Coefficient of variation was 5.12%.

Both, were inside the limits recommended for this type of assays (%).

Lowest Detectable Concentration.

The assay was able to detect amounts as low as 3 µg/g of CEA in fecal feces.

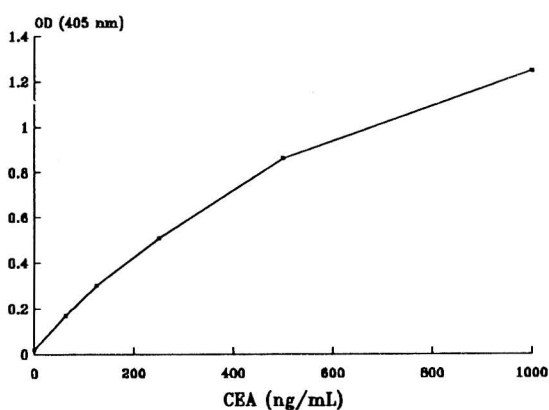


Fig. 1. Standard curve for CEA ELISA in fecal feces. Coating monoclonal antibody concentration: 15 g/mL, work dilution conjugate: 1:400. OD: Optical density.

Table 1

Intra assay variation. The mean, standard deviation and coefficient of variation represents the results obtained analyzing nine replicas per pool. Xm: Mean concentration, SD: Standard deviation, CV: Coefficient of variation.

DAYS	SAMPLES			
		A	B	C
1	Xm (ng/mL)	0.179	0.364	0.672
	SD	0.006	0.015	0.022
	% CV	3.59	4.19	3.4
2	Xm (ng/mL)	0.188	0.369	0.686
	SD	0.009	0.017	0.048
	% CV	5	4.81	7
3	Xm (ng/mL)	0.159	0.323	0.642
	SD	0.011	0.017	0.042
	% CV	7.2	5.4	6.69
4	Xm (ng/mL)	0.166	0.341	0.674
	SD	0.013	0.016	0.036
	% CV	7.9	4.8	5.3
5	Xm (ng/mL)	0.159	0.327	0.663
	SD	0.007	0.023	0.046
	% CV	4.56	7.33	6.9
6	Xm (ng/mL)	0.185	0.354	0.687
	SD	0.011	0.009	0.042
	% CV	6.3	2.78	6.2
7	Xm (ng/mL)	0.157	0.343	0.701
	SD	0.012	0.019	0.048
	% CV	7.8	5.5	6.85

Dilution Studies.

The curves obtained for the fresh samples and the standard are showed in figure 2.

A regression linear method was applied for each curve in order to achieve the best fit line and additionally, the coefficients "r" were calculated. For the samples an r mean value of 0.9934 was obtained and for the standard the calculated r was 0.9965.

The parallelism was analyzed by X² method. The mean X² calculated was 0.083 and significative differences were not found between the sample straight lines and the standard line with = 0.05.

Recovery.

The recovery of CEA in the enriched samples ranged from 95.1 to 111.9%. (Table 3).

Specimen Analysis.

Table 2.

Inter assay variation. The mean, standard deviation and coefficient of variation were obtained taking account the values of nine replicas per pool during seven days.

SAMPLES			
	A	B	C
Xm (ng/mL)	0.17	0.345	0.675
SD	0.013	0.017	0.019
% CV	7.64	4.92	2.81

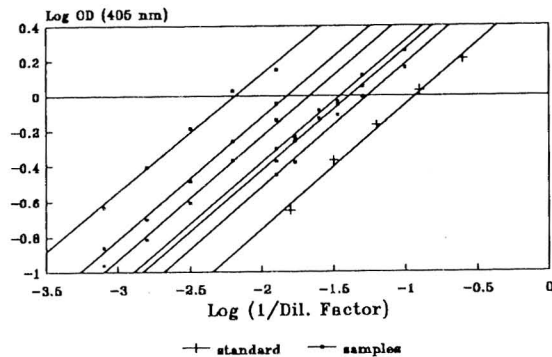


Fig. 2. Linearity dilution studies and parallelism. The six samples analyzed were linear with r mean value of 0.9934 was obtained. The r value for standard was 0.9965.

Table 3.

Recovery test. The recovery was analyzed through the formula: sample value + added amount = theoretical value; then, (measured value/theoretical value)100 = % recovery.

SAMPLE	Sample value ng/mL	Added value ng/mL	Measure value ng/mL	Theoretical value ng/mL	Recovery ratio %
A	292.2	40.03	316.13	332.23	95.15
B	93.62	40.03	140.4	133.65	105
C	35.13	40.03	82.13	75.16	109.2
D	686.43	40.03	724.4	726.46	99.7
E	205.9	40.03	269.83	245.93	109.7
F	882.9	40.03	1093	922.93	111.9
G	102.66	40.03	156	142.69	109.3

Table 4.

Means, standard deviations and subject numbers of the two analyzed population. It was found significant differences between these groups for an level of significance of 0.05, using the Mann-Whitney test.

n	Pathological Samples (μ g)	Control Samples (μ g)
MEAN	18	26
SD	144.26	69.9
	145.04	102.58

Table 4 shows the mean levels of CEA for the control subjects and colorectal patients.

Statistical significant differences were obtained between control and pathological samples with an U value of 331.5.

DISCUSSION

The performance characteristics of the designed ELISA system, such as the variation and accuracy assays fall between the expected values for this type of tests. This fact, guarantees the quality of the dosifications performed with the use of the system. Although, the unavailability of an authorized commercial test for the quantification of CEA present in human fecal feces disables us of a real validation of our system.

Nonetheless, the fecal standard for CEA was measured using an outlined system for quantification of CEA in human serum (Velandia *et al.*, report in press) which was compared with an international CEA standard and against the commercial Enzelsa CEA kit from CIS.

During the seventieth decade many reports describing the quantification of CEA in other fluids different from serum have been published. Sometimes rudimentary tests like gel-immunodiffusion method (Fred and Taylor, 1972) were used and certain differences between normal volunteers and neoplastic patients were found.

Furthermore, radioimmunoassay and ELISA methods were used in order to characterize different samples.

It is well known that the amount of CEA in the fluids present in the alimentary canal must be higher than the amount in the sera, for many reasons. One of them is CEA degradation that takes place in the liver. CEA low values in serum together with the tumor associated nature of the antigen have been some reasons that have not allowed the use of systems able to detect early colorectal cancer.

Fecal feces CEA level values higher than those reported in a previous serum CEA screening (Velandia *et al.*, report in press) were found.

Table 4 shown CEA levels in stool much higher than reported in a preliminary work by Fujimoto *et al.*, in 1978 (194.671.3 ng/g for colorectal cancer group and 7842 ng/g for control group). This difference could be due to the methodologies used for sample processing. In our study the fecal proteases activity was blocked using proteases inhibitors and the extraction step was mild without heating at 85 °C; method followed by Fujimoto, so, the possible denaturation of the molecule was avoided.

The use of specific monoclonal antibody for CEA immobilized on the plate prevents possible unspecific adsorption of CEA related substances like NCA-2 and normal fecal antigens, (Kuroki, *et al.*, 1981). A wider study including staged cases in the colorectal cancer

group is necessary to asses the usefulness for the early diagnosis of malignancies. The parallel determination of fecal and serum CEA could be a means of improving the predictive value of the determination.

REFERENCES

- ACEVEDO, G.; J. MARTUSCELLI; J. KUMATE; M. RUIZ; R. ORTEGA; M. LIEBERMAN. (1980). *Farmacopea de los Estados Unidos Mexicanos*. Quinta edición. Secretaria de salud. Mexico, p. 388.
- AVRAMEAS, S. (1968). Detection d' anticorps et d' antigènes á l'aide d' enzymes. *Bull. Soc. Chim. Biol.* 50, 1169.
- AVRAMEAS, S.; and T.; TERNYNCK. (1969). The cross-linking of protein with Glutaraldehyde and its Use for the Preparation of Immunoabsorbent. *Immunochemistry*. 6, 53.
- CASTELLI, M.; S. GUADAGNI; A. BAGNATO; M. A. PISTOIA; M. CARBONI; E. SEGA. (1986). Evaluation of Carcinoembryonic Antigen Levels in Gastric Juice, Stomach Mucosa and Plasma in High Risk and Gastric Cancer Patients. *Oncology*, 43, 149.
- FRED, D. L. J. and G. TAYLOR. (1972). Carcinoembryonic Antigen in Feces. *Br. Med. J.*, 8, 85.
- FUJIMOTO, S.; Y. KITSUKAWA and K. ITOH. (1978). Carcinoembryonic Antigen CEA in Gastric Juice or Feces as an Aid in the Diagnosis of Gastrointestinal Cancer. *Ann. Surg.* 189, 34.
- GOLD, P. and S. O. Freedman. (1965). Demonstration of Tumor-Specific Antigens in Human Colonic Carcinomata by Immunological Tolerance and Absorption Technique. *J. Exp. Med.*, 121, 439.
- HAMMARSTROM, S.; E. ENGVALL; G. SUNDBLAD. (1976). Carcinoembryonic Antigen (CEA): Purification, Structure and Antigenic Properties. *Scandia International Symposia. Health Control in Detection Cancer*. Almqvist & Wikson International. Stockholm. p. 32-39.
- KUROKI, M.; Y. KOGA; Y. MATSUOKA. (1981). Purification and Characterization of Carcinoembryonic Antigen-related Antigens in Normal Adult Feces. *Cancer Res.* 41,713.
- LAEMMLI, U. K. (1970). Cleavage of Structural Proteins During the Assembly of Bacteriophage T4. *Nature*, 227, 680.
- LOWRY, O. H.; N. J. ROSEBROUGH; A. L. FARR; R. K. RANDALL. (1951): Protein Measurement with the Folin-Phenol Reagent. *J. Biol. Chem.* 193, 265.
- LLANO, M.; E. CARPIO; R. ROSQUETE; R. SOLIS; M. GONZALEZ; J. GAVILONDO. (1989). Ensayo Diagnóstico para IgE humana por UltramicroELISA empleando Anticuerpos monoclonales. *Interferón y Biotecnología*, 6:2, 163.
- MANN H. B., AND D. R. WHITNEY. (1947). On a Test of Wether on of Two Random Variables Stochastically Larger than the Other. *Ann. Math. Statist.* 18, 50.
- OUCHTERLONY, O. and L. A., NIELSSON. (1978). Immunodifusion and Immunoelectrophoresis. *Handbook of Experimental Immunology*. D. M. Weir. Blackwell Scientific Publication. Oxford, London, Edinburg, Melbourne. p. 196.
- SMITH, S. J., G. R. COOPER, L. O. HENDERSON, W. H. HANNON, (1987). International Colaborative Standardization of Apolipoproteins A-I and B. Part I. Evaluation of a Lyophilized Candidate Reference and Calibration Material. *Clin. Chem.* 33, 2240.
- SNEDECOR, G. W., W. G. COCHRAN. (1980): *Statistical Methods*, 7th edition. Iowa State University Press. Iowa, p. 238-254.
- SUGARBAKER, P. H. (1976). Carcinoembryonic Antigen (CEA) Assays in Obstructive Colorectal Cancer. *Ann. Surg.*, 184, 752.
- TATSUTA, M., T. ITHO, S. OKUDA, H. YAMAMURA, M. BABA, H. TAMURA. (1980). Carcinoembryonic Antigen in Gastric Juice as an Aid in Diagnosis of Early Gastric Cancer. *Cancer* 46, 2686.
- TATSUTA, M., H. YAMAMURA, R. YAMAMOTO, T. MORII, S. OKUDA, H. TAMURA. (1982). Carcinoembryonic Antigen in the Bili in Patients with Pancreatic and Liliary Cancer. *Cancer* 50, 2903.
- TORMO, B. R., J. V. GAVILONDO, C. DOMINGUEZ, M. FREYRE, T. RODRIGUEZ, P. BIBERFELD. (1989). CEA in Colonic Adenocarcinomas and Precancerous Lesions. *APMIS* 97, 1073.
- VELANDIA, A., G. NUNEZ, R. ROBAINA. Ultramicroanálisis para la cuantificación de Antígeno carcinoembrionario en suero. *Biotecnología aplicada*. (Report in press).