Revisiting the dilution factor as vital parameter for sensitivity of ELISA assay in CSF and Plasma

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KEY WORDS

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ABSTRACT

Background: **Enzyme Linked Immunosorbent Assay** (ELISA) is very sensitive assay which provides quantitative data about expression of antigens. However, its utility is based on certain parameters which vary in the experimental situations.
Purpose: We aimed to analyse the dilution factor as an important parameter for determining the sensitivity of ELISA in human samples.
Methods: Total of n = 57 ALS patients and n = 48 normal controls were selected for the study. All the patients were recruited from, Department for Neurology and Anaesthesia, PGIMER. Blood and CSF sample was collected and ELISA run was performed in both plasma and blood sample. ELISA of OPTN and TDP-43 was employed to check the respective protein concentration in CSF and Plasma.
Results: There was no significant difference which was reported for Plasma as well as CSF values of TDP-43 and OPTN. Dilution test prior to actual experiment made a significant impact in deciding the actual concentration of sample and led to overshootingbeyond range of reference protein.
Conclusion: Negative results from our study highlights the significance of determining the dilution factor as an important parameter for conduct of ELISA.

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Introduction

ELISA assay is one of the reliable and efficient techniques in the field of immunology. Working of antigen antibody affinity exploits the natural properties of antibody binding and quantify the results on the basis of color emission from the reaction solution. This technique finds its application in detecting the protein levels in almost all available biological tissues ranging from plasma, serum, tissue homogenates to culture studies. However, what makes this technique reliable is the steps involved in carrying out the experiment. Optimum concentration of target protein and antibody makes up an important parameter for obtaining better results. In 1977, a group from Atlanta, Georgia US came up with a standard protocol for quantifying ELISA.[1] Standard curve is an essence of every ELISA experiment. It helps in determining the true concentration of target protein in different samples. Several studies have claimed that even minimal errors in standard curve can impact the experimental outcome.[2,3] Although the result for protein titre values depend on standard curves, however, the quality of standard curve is dependent on dilution.

Dilution is a vital parameter for ELISA experiment which in turn determines the values of detection range for antibody and target antigen concentrations. Even the standard curves which are plotted in every ELISA run has a varying sets of results indicating that same type of ELISA experiment with respect to standard curve done at different time points give different results.[4,5] Depending on dilution the normal method of interpreting a result is through Optical Density (OD) obtained at different concentrations of standard. This OD to dilution correlation is determined by the hyperbolic curve function. More the linearity more is the stronger correlation between the OD and standard concentration.[3,6] However, it is not absolutely necessary to obtain the straight line every time we perform an ELISA assay. There are certain situations when the value of correlation coefficient r^2 may approximate 0.98 (which is considered as an optimum value of straight line).[7]

In current study we present a data to understand the impact of dilution factor on ELISA results. We used cerebral spinal fluid (CSF) and plasma samples from both Amyotrophic Lateral Sclerosis patients as well as controls. As CSF is in direct contact to brain, hence it may be used to understand the inflammation in case of degenerative diseases. Some of the studies in last 3 decades describe the lower concentration of proteins in CSF.[8,9] However, due to low concentration of protein in CSF the ELISA the quality of CSF in terms of sensitivity for protein concentration cannot be neglected. Low protein concentrations certainly make CSF assay more sensitive in diagnosing the disease onset.[9,10] We also used plasma to analyse protein concentration in addition CSF due to increased protein levels.[11,12] Both CSF and plasma have been studied extensively in brain related disorders[11–15] and proved to be an dependable source for use in diagnosis ofbrain disorders.

Methods

Subject recruitment

All the ALS (n = 17 for CSF, n = 40 for plasma) patients were recruited from Out Patient Department of Neurology, PGIMER, Chandigarh, INDIA between 2011 to 2013. All the patients were diagnosed for ALS after confirming the El Escorial criteria and Functional Rating Scoring (FRS). Informed consent was obtained from patients before taking sample. The study was approved by institute ethical committee. Below is the table for inclusion and exclusion criteria for the patients of study.

Table 1: Inclusion and exclusion criteria for both patients and controls of the study

S.No	ALS Patients		Non Neurological Controls	
	Inclusion	**Exclusion**	**Inclusion**	**Exclusion**
1.	Diagnosis established following the World Federation of Neurology criteria or El escorial criteria	Patients not fit valid for El escorial criteria	Mean Age of control 20–65 years.	Age below 20 years
2.	Altered electromyography features	Intake of medicines other than angiotensin-converting enzyme inhibitors, beta blockers, dietary supplements, vitamins, alendronate and methylphenidate. Steroids (and medicines prescribed with them such as calcium supplements and proton pump inhibitors) will be discussed	Absence of Muscular weakness	Concomitant chronic or acute muscular, neurological (including mental retardation and autism), infectious or inflammatory disorder in the three weeks preceding the blood test
3.	Progressive muscle weakness	Patients with cognitive impairment with significant decision making incapacity, or major depression, or schizophrenia, or dementia (e.g. Alzheimer's disease or Subjects with uncorrected hypothyroidism or hyperthyroidism	Absence of Infection	Vaccination or treatment with immunoglobulin's within the three months preceding inclusion
4.	Medullar onset of the disease	Treatment with corticosteroids, immunoglobulins or immunosuppressors during the last 12 months		Informed consent not signed.
5.	ALSFRS from 23 to 43	History of bleeding disorder, which would make a blood draw unsafe		
6.	Subjects taking Riluzole must have been at a stable dose for at least 30 days with no evidence of toxicity	Pre-cancerous conditions (e.g. Barrett's Esophagus, dysplasias) or benign tumors which have the potential for significant growth due to VEGF stimulation		
7.	Age over 20 years and below 65 years	Age below20 and above 65 years		
8.	Parents or if applicable subjects must give informed consent			
9.		Evidence of chronic or active heart, liver, kidney, or lung diseases, or Age-related macular degeneration.		
		Female subjects who are either pregnant or nursing. Bladder or bowel involvement. Extra ocular involvement		

TDP-43 and OPTN analysis

According to existing literature, wildtype OPTN is believed to have a role in reducing the apoptosis in neuronal cells by moderating TDP-43 levels.[16,17] ELISA assay was performed to quantify the levels of TDP-43 and OPTN in CSF as well as TDP-43 in Plasma. ELISA assay was carried out as per manufacturer's protocol (Blue gene Elisa Kit).

Plasma isolation

8.0 mL blood was collected in an Ethylenediaminetetraacetate (EDTA) tube and kept at room temperature for ~2-3 hrs. Upper yellowish portion was collected and layered on equal volume of Histopaque. After centrifugation at 1800 rpm for 30 mins, plasma was collected in separate vial and stored in -80^{0}C until used.

Dilution factors in different assays

Undiluted samples were used in case of both CSF and plasma ELISA assay. Both TDP-43 and OPTN proteins were quantified in CSF and plasma. However, in case of plasma, prior to ELISA, a limited 8 well ELISA based dilution factor test was run for 0X, 10X, 100X and 500X plasma samples so that the best dilution factor could be obtained for the entire experiment. Based on obtained values undiluted concentration of plasma samples were used.

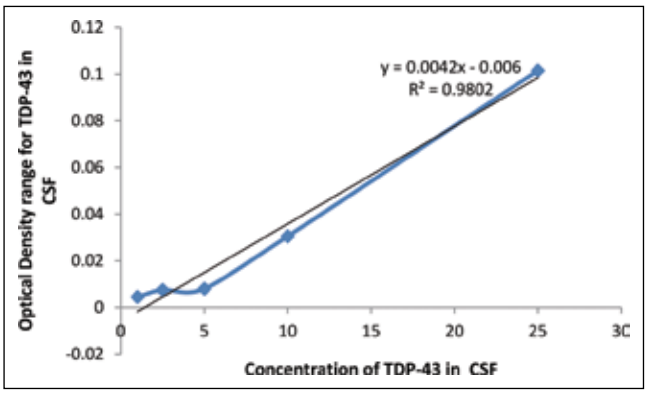

Fig. 1: R^2 showing correlation between OD and concentration in TDP-43 CSF.

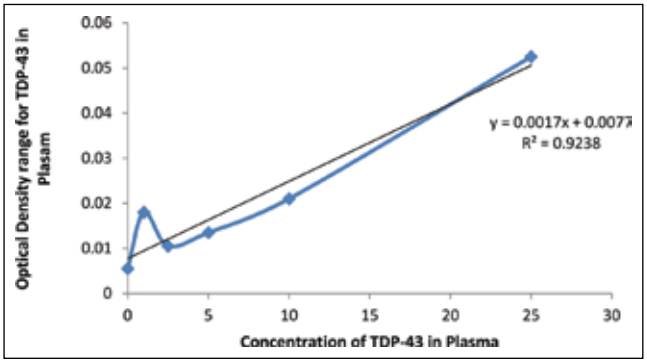

Fig. 2: R^2 showing correlation between OD and concentration in TDP-43 Plasma.

Results

TDP-43 in CSF and Plasma

The standard range varies from -0.001 to 0.10115 (in TDP-43 CSF), 0.0055 to 0.0525 (in TDP-43 plasma) for variations in concentration between 0 ng/ml to 25 ng/ml (TDP-43 in CSF and palsma). A linear trend fits well to the values of concentration and OD with R^2 = 0.980 and 0.923 for (TDP-43 in CSF and TDP-43 in plasma) (Figure 1 and 2) and linear prediction as OD = 0.0042(Conc) - 0.006 in case of CSF and OD = 0.0017(Conc) + 0.0077 in case of plasma.

Based on above equations the concentration was computed for 18 ALS patients in case of CSF and 40 in case of plasma. It has been observed that most of concentration values fall outside the standard range and this may be due to absence of dilution factor (Figure 3a, b, c, d).

Boxplot for control and ALS patient given in figure (4) for TDP-43, shows that the data is not normally distributed for control as well as ALS (in both cases it is negatively skewed).

OPTN in CSF

Similarly, in case of OPTN in CSF the standard range varies from -1.1945 to 0.126 for variations in concentration between 0 ng/ml to10 ng/ml. A cubic trend fits well to the values of concentration and OD with R^2 = 0.983 and the prediction equation as Conc. = -14.49(O.D.)^3 - 13.86(O.D.)^2 - 3.392(O.D.) + 0.628 (Figure 6).

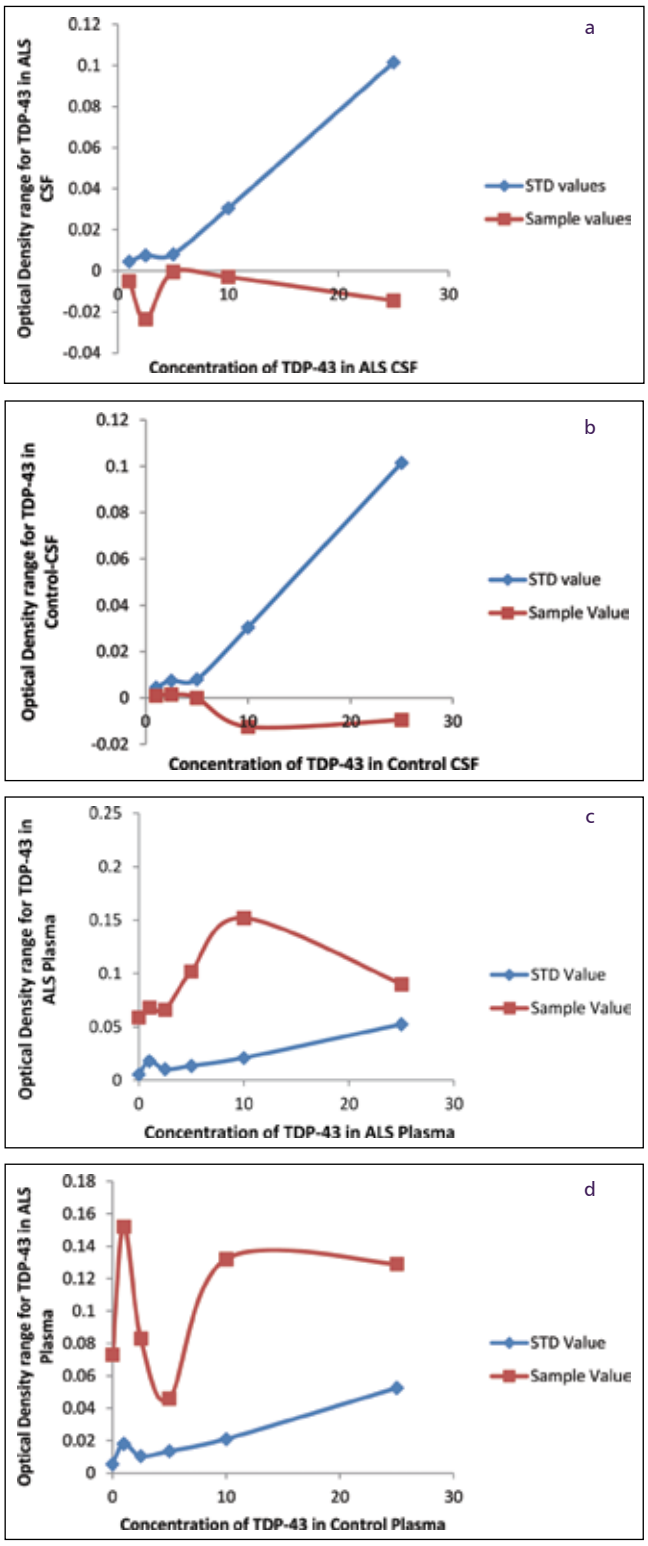

Fig. 3: Red line representing the OD values of actual sample falling beyond the range of standard in (a), (c) ALS(CSF and Plasma respectively), (b) (d) Control (CSF and Plasma respectively).

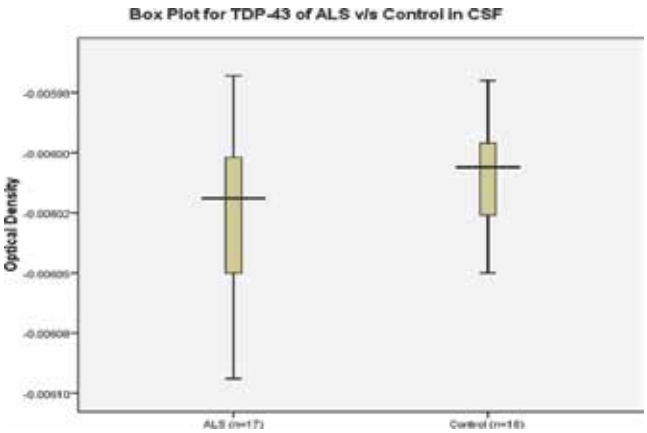

Fig. 4: Box plot showing distribution of TDP-43 among ALS patients and controls in CSF.

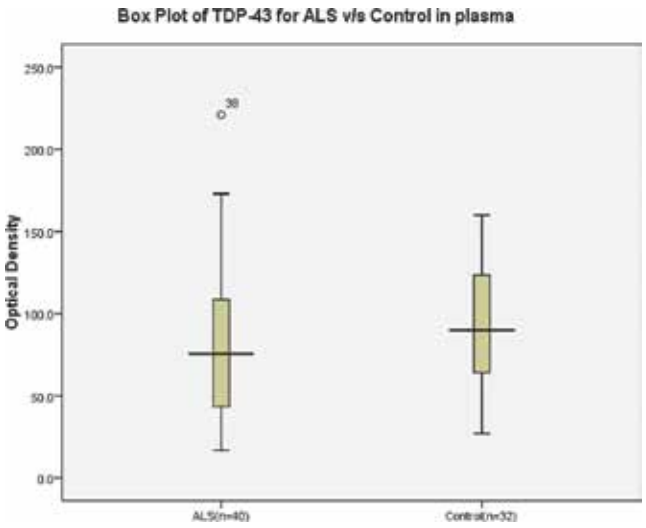

Fig. 5: Box plot showing distribution of TDP-43 among ALS patients and controls in plasma (althogh normally distributed for ALS but not for Control).

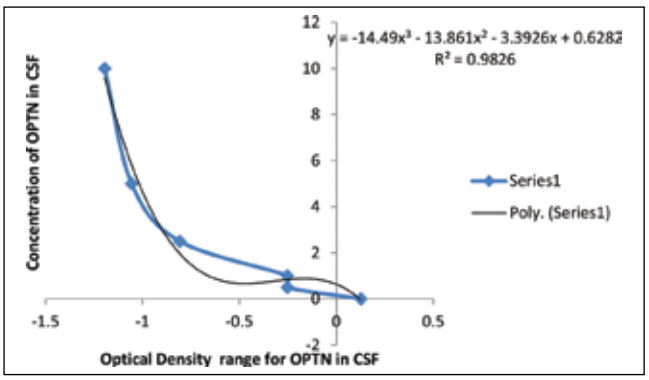

Fig. 6: R^2 showing correlation between OD and concentration OPTN CSF.

Based on this equation the concentration was computed for 17 ALS patients v/s 16 controls.

Similarly, it was observed that most of concentration values again fall outside the standard range and this may be due to absence of dilution factor (Figure 7a, b).

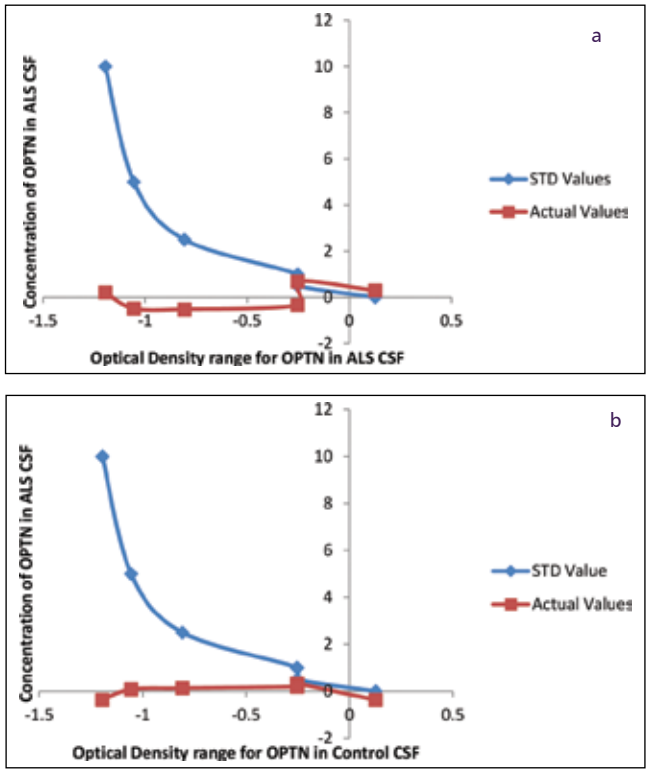

Fig. 7: Red line representing the OD values of actual sample falling beyond the range of standard in (a) ALS (b) Control.

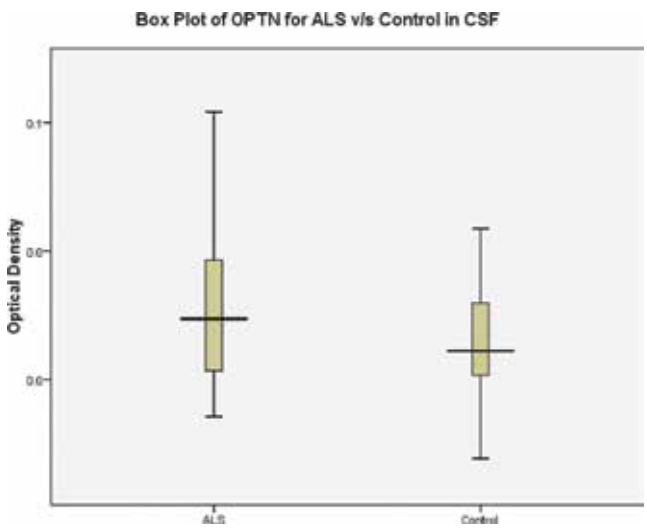

Fig. 8: Box plot showing distribution of OPTN among ALS patients and controls in CSF.

Boxplot for control and ALS patient given in figure (5) shows that the data is not normaly distributed for control as well as ALS. Figure 8.

The ALS and Control groups were compared using Mann Whitney-U statistics and the results are presented in Table 2.

Discussion

Current study describes the failure of experiment conducted to analyse protein concentration of TDP-43 and OPTN through

Table 2: Mann whitney test statistics along with p-value to compare levels of TDP-43 CSF, TDP-43 Plasma and OPTN CSFin Control and ALS

Molecule	Control Mean Rank	ALS Mean Rank	Wilcoxon (Z-Statistics)	p-value
TDP-43 CSF	21.00	15.17	–1.685	0.092
TDP-43 Plasma	40.12	33.60	–1.315	0.189
OPTN CSF	15.82	20.06	–1.221	0.222

Table 3: Readings of dilution assay done for plasma sample prior to ELISA experiment

STD Conc ng/ml	OD for STD Conc	Dilution Factor	OD for Diluted samples
2.5	0.136	500X	0.141
5	0.155	100X	0.138
10	0.162	10X	0.136
25	0.17	0X	0.16

ELISA in CSF and plasma samples of ALS patients and highlights the importance of dilution as an important parameter to estimate the protein titers in CSF and Plasma both of which are considered to be sensitive tests for determining protein concentrations. Figures 1, 2 shows the value for standard curve which corresponds to 0.980 and 0.923. These results conform with previous study which describes the value for linear regression coefficient to be near 0.98.[7] Graphs from figure 3a, b, c, d and 7a, b show the position of actual values for test and control samples and value of standard recombinant protein of TDP-43 and OPTN in both CSF and plasma. A difference can easily be made between the value range of both sample and standard. Since our values were beyond the range of standard hence, we applied non parametric Mann Whitney test to analyse the normal distribution of TDP-43 and OPTN levels in bot CSF and plasma of ALS and control. Table 2 summarizes the result of Mann Whitney test by showing the comparison between the means of all protein concentration in CSF and plasma of sample and control. The results from our study show the importance of standard curve and dilution as an important parameter for quantifying the ELISA results. While there are certain cases in which the sample values falling in standard curve OD readings correspond to correct results of ELISA assay contrary to what we have obtained in this study.[18–25] Thus the negative results in current study validates the earlier studies which supports the use of dilution as an important parameter to analyse the protein concentration of unknown target protein,[26–29] contrary to few studies which report the values of ELISA assay with undiluted samples in case of CSF.[30,31] Our result is in continuation with the studies which describe the role of different time intervalsin plating a standard for ELISA which may end up in different results.[4–6] Dilution factor before ELISA is a crucial step before final anaysis. A dilution assay informs the amount of actual concentration that should be taken from sample to conduct an experiment. In current study, absence of dilution assay (in case of CSF experiments) and critical analysis of dilution (in case of Plasma experiment) led to inconclusive results. In Table 3 we present a result of dilution assay done for plasma sample prior to ELISA set up. Here, OD against 0X dilution of sample corresponds to a range beyond 10 ng/ml of standard. However, if carefully analysed, OD value ranging between dilution factor 0X to 10X could be an actual choice of dilution. Hence, if the experiment would have been conducted at 5X dilution which lies between 0X and 10X, values should have fallen within the range of standard.

Owing to the dilution factor, these parameter make up the vital part of any ELISA experiment. Without optimum values for dilution, highly sensitive results for protein concentration cannot be achieved. In order to achieve valid results one has to keep an essential high alert check as if standardization of dilution goes wrong values are going to offshoot beyond the detectable range of standard.

Authorship contribution

Akshay Anand: Conceptualised and designed the study, **Sudesh Prabhakar:** Provided the ALS patients, **Pawan Gupta:** Collected the control sample, **Keshav Thakur:** Acquired data, conducted the experiment, **Suresh Sharma:** performed the statistical analysis.

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