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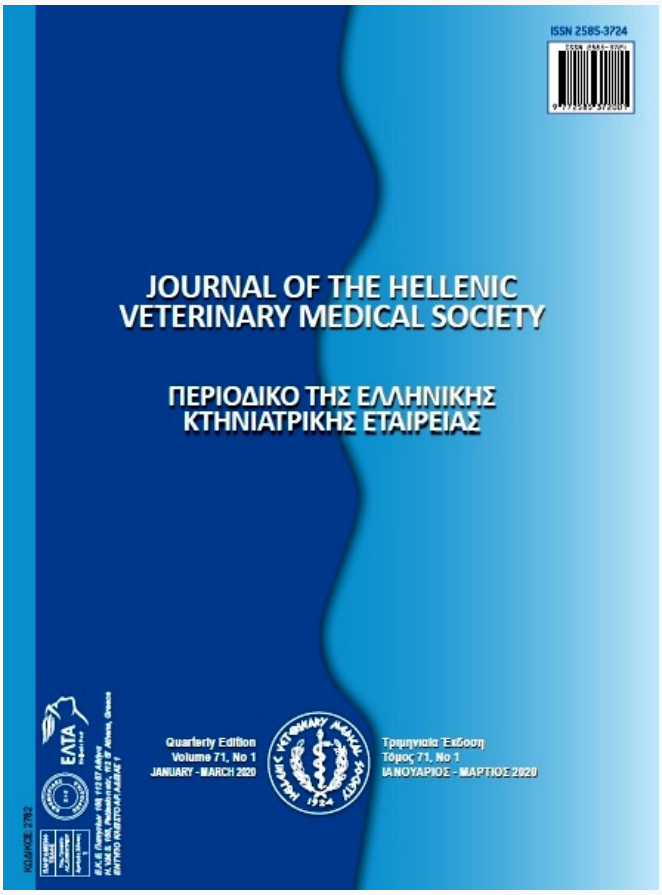

Development of Enzyme Linked Immunosorbent Assay for humoral immuneresponse and infection monitoring of anthrax

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Research article
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Development of Enzyme Linked Immunosorbent Assay for humoral immune response and infection monitoring of anthrax

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ABSTRACT. Immune assays were taken into consideration to diagnose and quantify metabolites such as antigen and antibody. Enzyme-Linked Immunosorbent Assays (ELISAs), which are used to detect antigens and antibodies, generated several periods of infectious and vaccination conditions. There is an extensive range of commercial infectious disease ELISA kits useful for the detection of human and animal IgG, IgA, IgM antibodies and microorganism antigens. Anthrax is one of the serious infectious diseases caused by rod-shaped, gram-positive bacteria known as *Bacillus anthracis*. Subunit or attenuated vaccines applied against anthrax disease increase the antibody against the Protective Antigen (PA) which has a critical role as a toxin of *B. anthracis*. Herein, the ELISA was developed using PA domain 4 and anthrax Lethal Factor to detect IgG antibody in serum. Besides, the level of anti-LF antibodies were determined as a complementary test to measure variance in antibody titers associated with vaccination or infection that leads to detection of anthrax in livestock. The results show that we developed high-quality ELISA kit that can be used to test immunogenicity of vaccines and infections in mice. We tried to develop the Anti- PA4 ELISA kit and conduct the validation studies to evaluate the fluctuation level of the antibody in the anthrax vaccine and distinction between disease and vaccination in mice.

Keywords: PA4; Anthrax; Humoral immunity; validation; ELISA.

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INTRODUCTION

Enzyme-linked Immunosorbent Assays (ELISAs) as an immune assays approach are applied to detect antigens and antibodies throughout the infectious and vaccination situation. Nowadays, a broad range of commercial ELISA kits is being utilized to detect human and animal IgG, IgA, IgM antibodies and microorganism antigens. Since its discovery 80 years ago, the live attenuated Sterne strain (34F2) of *B. anthracis* has been successfully used as the predominant method to immunize livestock against anthrax, leading to the control of several positive cases of anthrax in different species (Tadayon et al., 2016). The mechanism employed by such vaccines is to trigger the immune system against the protective antigen (PA) of the *B. anthracis* (Dumas et al., 2017). Anthrax toxin is composed of protective antigen (PA), lethal factor (LF) and edema factor (EF). All of which play a significant role in boosting the immune system's response to such vaccines; however, the PA (among the three component PA, LF and EF) is the most effective factor in increasing the immunogenicity in *B. anthracis* toxin (Zai et al., 2016). Although the existing vaccines such as AVA are safe and effective, there has been a surge in the need for anthrax vaccines due to the increased terroristic attacks in the recent decades. Furthermore, the prevalence of such bacteria among humans has highlighted the necessity of research and developing methods of producing subunit vaccines (Malik et al., 2018). PA is one of the most important antigens in the anthrax which is used in the design of vaccines and several commercial diagnostic kits. It plays a key role in the pathogenesis of *B. anthracis*, and if the antibody against PA is produced, it can prevent the binding of PA to the cell surface, and eventually the function of *B. anthracis* toxin will be impaired{Male, 2017 #109} (Goldsteinet al., 2017). The anthrax PA is a four-domain protein that each part plays a specific role (Male et al., 2017). The fourth domain maintains the structure and binding to the host receptor Capillary morphogenesis protein 2 (CMG2) which leads PA to bind to the cell. So the PA domain 4 (PA4) has a critical role in the function of PA and the toxin of *B. anthracis* (Mamillapalli et al., 2017). Subunit vaccines or attenuated vaccines applied against anthrax disease increase the immune systems' antibody against the PA. Therefore, the cornerstone of a vaccine showing its protection and efficacy in curbing the disease is the power of antibody applied against the PA (Sim et al., 2017). {Sim, 2017 #113}

Testing the potency of anthrax vaccine entails using a virulent strain of *B. anthracis* so that the real immunization of the vaccine would be determined (Moazeni et al., 2007). Conducting bioassay and lethal challenge on laboratory animals with the virulent strain entails the use of specialized containment equipment including class-3 facilities, proper environment and special laboratory tools. Such facilities are not available everywhere (Milleret al., 2012). On the other hand, testing the immunization of a vaccine in field and clinical studies entails the adoption of a safe and simple method (Ionin et al., 2013). The existing ELISA kits measure the practicality of antibodies against PA. The amount of antibody produced against PA shows the stimulated cases, (human or animal) possibility of exposure to the microorganism or the vaccination (Laws et al., 2016). However, determination of the subjects' exposure to the microbe or vaccine is not possible with the available ELISA kits.

Studies show that the most specific antibody against the *B. anthracis* is the antibody produced against the PA4. That is because the generated anti-PA4 prevents PA's binding to the receptors in the cell surface and consequently prohibits the LF and EF from entering the cell and causing the disease. Therefore, measuring the titer of anti-PA4 following the injection of PA4 to mice can provide a safe and simple method to show the potency of such vaccines (Williamson et al, 2015). On the other hand, the use of the PA4 in the ELISA kit design can be indicative of an effective immune response against the disease as compared to other commercial types.

The present study tries to use the new recombinant antigen PA4 to survey the pattern of humoral immunity responses kinetically. It is done after the injection of AVA, Razi anthrax vaccine and PA4 antigen with aluminum hydroxide adjuvant. In order to differentiate the infection caused by the *B. anthracis* and the immunity obtained from injecting several anthrax vaccine, virulent strain *B. anthracis* (17JB) with a sub lethal dose was used to survey the rise of produced antibody against PA4 and LF. The Anti-PA4 ELISA was developed and the validation studies were conducted to evaluate the antibody titer in the anthrax vaccine in mice. The validation purpose of this approach is to show that such method suits our study. All of the needed instructions have been followed to validate the study based on the international Conference of harmonization (ICH). (Pombo et al., 2004).

MATERIALS AND METHODS

Antigens and Vaccines

The new recombinant PA4 and the LF antigens were supplied from Razi Vaccine and Serum Research Institute's Immunology Department (Karaj, Iran) and kept at the temperature of -70 °c. The AVA vaccine was supplied from Biothrax Company. The Razi anthrax vaccine, which is a *B. anthracis* live spore vaccine, and the *B. anthracis* strain 17JB were supplied from Razi Vaccine and Serum Research Institute.

Reference Serum

The domestic reference serum dubbed 'Pool' used in the research as the PA4 antigen (25 µg) was mixed with the aluminum hydroxide adjuvant 2.5% V/V, which was diluted 1/10 with PBS, and 0.25mL of the mixture was injected subcutaneously to ten mice Balb/c in the timespan of thirty days. The blood sampling was conducted in the weeks 1, 2, 4, 6, 8, and 10 and the serum was collected afterwards. The serum attained from 3 mice with the most OD (>2.5) during those dates was selected and mixed together. The serum pool of its antibody was conventionally set as 800 EU/mL. It was divided into 0.2 mL micro tubes and kept at the temperature of -70 °C (Pombo et al., 2004).

Samples

Experimental Balb/C mice were allocated into five groups, each containing ten animals. Mice of the first three groups were treated in the days 1 and 15 with 0.25mL AVA vaccine, 0.25mL of The Razi Anthrax vaccine and 25µg PA4 antigen with aluminum hydroxide adjuvant, respectively. The fourth group contains the virulent strain of *B. anthracis* (17JB) in which 150 *B. anthracis* live spore was injected subcutaneously; the sampling was carried out on third and fifth weeks. For the last group, Normal Mouse Serum (NMS) was applied to be used as a negative control (Xiang et al., 1994). All serums kept at the temperature of -70 °C. All experimental protocols were conducted according to the principles described in guidelines for care and use of laboratory animals and approved by the Research and Ethics Committee of Babol University of Medical Sciences (MUBABOL.REC.1395.175).

ELISA for anti-PA4 and anti-LF antibody measurement

A checkerboard titration was carried out to settle the optimum antigen concentration and conjugation was diluted by blocking buffer just before being used in the ELISA. Antigen concentrations considered in the study included 200 ng/well of PA4 and 100 ng/well LF in 0.05 M Carbonate-Bicarbonate, and pH 9.6 that were coated on 96 well ELISA plates (Jet bio, Canada) and incubated overnight for 24 h at 4°C (amount of proteins were determined by Bradford assay). Plates were washed once with phosphate buffered saline (PBS) and then they were blocked with 200 µL/well of 3% skimmed milk (Sigma) for 24 h at 4°C. After washing once with PBS, the plates were incubated with 100 µl/well controls and test samples at a dilution of 1/80 in the dilution buffer (3% skimmed milk) for 2 h at 37°C. Each serum sample was tested in triplicate. The wells were again washed for four times with PBS and incubated with 100 µL/well Anti-mouse IgG HRP (Sigma USA) diluted to 1:40,000 in the dilution buffer and incubated at 37°C for 2h. Finally, washed for five times with PBS, unbound antibodies were removed and the bound antibodies were detected virtually after 15-20 minutes in the dark using 100µl of 3,3',5,5'-tetramethylbenzidine (TMB), containing hydrogen peroxide (Sigma) as the substrate along with H_2O_2. The reaction was stopped by adding 50µl of HCL 5.8% and absorbance was read in wavelength 450 nm against 630 nm second filters in an ELISA reader (Stat Fax. USA) and ELISA value was obtained (Ndumnego OC et al., 2013). All washing steps were carried out with micro plate washer (STAT FAX, USA).

Linearity

A total of seven serum samples of varying antibody concentrations, S1 through S7, were used for the assessment of linearity. The S1 is the domestic reference serum 'Pool'. Of the S1, the samples S2 through S7 were attained with dilutions of 1/2 to 1/64. The NMS was used for the dilution of all samples. All samples, S1 to S7, were put in three different plates in triplicate. As per protocol, the amount of anti-PA4 in all samples was obtained based on optical density (OD). The curve linearity was obtained from the analysis of the empirically observed log10 amount of antibody in the samples and finally was reported as EU/mL. Through the attained results, the P-value, the correlation coefficient, the y-intercept and the slope of the regression line were calculated (Semenova et al., 2017).

Accuracy

To perform the accuracy test, the samples S1, S3, S5 and S7 were used. In this test, coating was carried

out according to paragraph 2.5; the amount of anti-PA4 was assessed based on OD and reported as EU/mL. The accuracy is expressed as percent recovery and is calculated with the following formula: Mean estimated unitage per mL / assigned unitage per mL × 100.

The calculated concentration from each sample shows the average geometrical estimation based on the S1 which is corrected by the dilution of each sample (Pombo et al., 2004).

Precision

The assessment of repeatability (Intra-plate, Inter-plate, and Intra-day) and intermediate precision between the days and analysts was conducted according to the definitions ICH. Precision was determined using three samples namely S1, S3 and S5 which were in accordance with linearity tests. In this stage, coating was also conducted according to paragraph 2.5. Based on the protocol, the amount of anti-PA4 was measured independently by two analysts in three consecutive days and then the average OD was assessed for each sample. Finally, the results were expressed in EU/mL and the results of OD for each sample were calculated by %CV (SD/mean×100) (Semenova et al., 2012).

Quantification Limit (LOQ)

To perform this test, three samples namely S5, S6 and S7, which indicated a low concentration of the antibody, were used. Coating was conducted according to paragraph 2.5. According to the protocol, the amount of anti-PA4 was measured based on OD in three non-consecutive days within an eight-day period. The average OD was calculated for each sample and at the end, the antibody was reported by EU/mL for each sample. The results of OD were assessed by %CV (SD/mean×100) (Ghosh et al., 2015).

Detection Limit (LOD)

Detection limit (LOD) was obtained using two NMS serums with the dilution of 1/80 and each sample was divided into two different plates in triplicate. Anti-PA4 was measured based on OD according to ELISA protocol in five non-consecutive days in a two-week period. The LOD was estimated by interpolating the mean of all 68 OD values, plus standard deviations. The LOD was calculated by the following formula: Mean (OD) + 3SD; then, it was reported in EU/mL (Ghosh et al., 2015).

Specificity

To perform this test, besides the Pool and NMS serums, other serums namely Foot-and-mouth Disease (FMD), Pasteurella (PAS), Agalactia (AG) and diphtheria pertussis (whooping cough) and tetanus (DTP), which were obtained in previous studies, were used. Three different dilutions (1/80, 1/160 and 1/320) were prepared from each sample using the NMS serum. The serums were put in two separate plates and ELISA value was obtained. Only one dilution (1/80) was used for NMS.

Competitive Inhibition ELISA

To determine the specificity of the measurements performed by ELISA, competitive inhibition ELISA (CI-ELISA) has been developed based on the qualified anti-PA4 IgG ELISA. The CI-ELISA was performed by using following modifications of the standard ELISA procedure. 96-well polystyrene plates (Jet bio, Canada) were coated at 4°C for 24h with 200 ng/well PA4 antigen in 0.05 M carbonate-bicarbonate buffer (pH 9.6) per well. The plate was washed once with phosphate buffered saline (PBS). After the blocking with 200 µl skimmed milk %3 at 4°C for 24h and washing once, 100 µl of 1:80 dilution serum and 780 pg to 800ng of PA4 were added and incubated at 37°C for 2h. limiting concentration was determined empirically by the titration of anti-PA4 in standard ELISA. The wells were washed four times with PBS and incubated with HRP anti-mouse immunoglobulin G (Sigma, USA.) at 37ºC for 2h. Finally, wells were washed five times with PBS, 100µ of substrate buffer containing TMB per mL was added, and the mixture was incubated for 15-20 min. In the follow-up step of ELISA, reactions were stopped by adding 50µl of Hcl5.8%, and the optical density (OD) at 450 nm and 630 was determined and ELISA value was obtained. (Ndumnego et al., 2013; Mitic et al., 2016).

Comparing the amount of antibody against PA4 and LF antigens in the vaccinated mice with the mice injected with virulent strains of *B. anthracis*

The two antigens PA4 (200ng) and LF (100ng) were coated in two separate plates to compare the antibody against the LF and PA4 in sera of the vaccinated mice and the mice injected with virulent strains of *B. anthracis*. Tests were performed in duplicate.

RESULTS

Performance Characteristics of the Anti-PA4 IgG ELISA

The assessment of Antibody development pattern proved that all vaccinated mice had anti-PA4 as determined by ELISA, with ELISA values which gradually increased until week 4, then slowly declined (Figure. 1). Antibodies were detectable by 2 to 4 weeks after vaccination (Figure. 1). Sera from the NMS did not develop titers by ELISA (Figure.1).

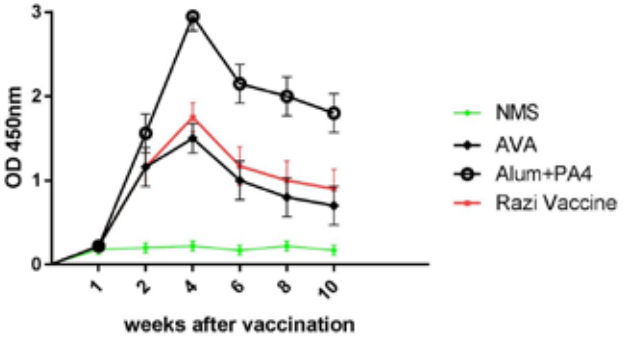

Fig. 1. Antibody levels in vaccinated and NMS mice as determined by ELSA. Open symbols indicate vaccinated mice, and solid symbols indicate NMS mice. Vaccinated mice were inoculated with PA4 antigen plus adjuvants, Razi Vaccine and AVA subcutaneously.

Linearity

After calculating the log10, the obtained OD values from all of the samples, S1 through S7, showed that the curve in the range 14-892 EU/mL was completely linear and the values showed to be as follows: r^2 = 0.9628, *P- value* < 0.005 and Y= ax + b (Y= 0.9601x+b) (Figure.2).

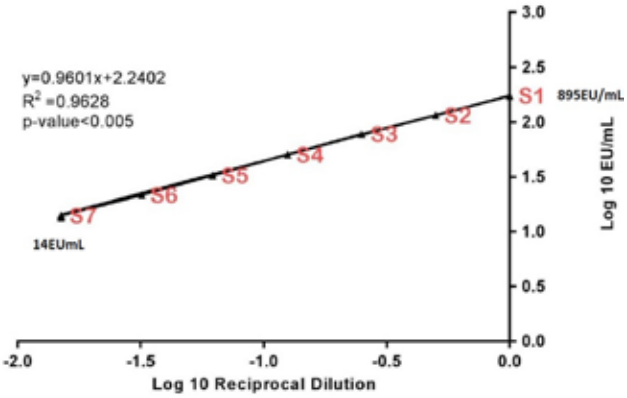

Fig. 2. Linearity curve. EU/mL of seven dilutions of S sera (the pool as domestic reference serum) are plotted against varied dilution (1:2, 1:4, 1:8, 1:16, 1:32, and 1:64). Each point represents the estimated EU / mL of each unit that was tested three times.

Accuracy

The accuracy results in Table-1 are stated in the form of percent recovery. To perform the test, the range between 16-1000 EU/mL was studied and the percent recovery came out to be between 90-117%. The resulted percent fits the validation criteria according to Table 2.

Table 1. Accuracy assessment. The accuracy results are stated in the form of percent recovery.

	Replicates	Target sample (EU/mL)			
		S1 (1000) % Recovery	S3 (250) % Recovery	S5 (62.5) % Recovery	S7 (16) % Recovery
Plate 1	1	94.3	100	82	110
	2	95	101	84	117
	3	112	102	93	120
Plate 2	1	104	100	90	130
	2	110	108	92	120
	3	108	110	90	114
Plate 3	1	93	103	90	130
	2	103	100	103	100
	3	102	103	89	110
Mean		102.36	103	90.33	116.77

Table 2 .Acceptance criteria and characteristics of the anti-PA4 mouse ELISA. To perform the test, the range between 16-1000 EU/mL was studied and the percent recovery came out to be between 90-117%.

Assay characteristic	Acceptance criteria	Results
Prescision-repeatability	%CV < 20	%CV ≤ 13.7
Intra-plate		%CV ≤ 15
Inter-plate		%CV ≤ 12
Intra-day		
Intermediate prescision	%CV ≤ 25	%CV ≤ 18
Days	%CV ≤ 30	%CV ≤ 7
Analysts		
Accuracy	80 - 120% Recovery	90 - 117% Recovery
Limit of detection (LOD)	-	7 EU/mL
Limit of quantitation (LOQ)	-	14 EU/mL
Linearity	-	Working range: 27 - 892 EU/mL $r^2 = 0.96$

Table 3. Specificity. To perform this test, besides the Pool and NMS serums, other serums namely Foot-and-mouth Disease (FMD), Pasteurella (PAS), Agalactia (AG) and diphtheria pertussis (whooping cough) and tetanus (DTP) were used.

Sample Pool	**Dilution in well**	**Elisa values**	
		Plate 1	**Plate 2**
	1:80	99.192	92.419
	1:160	77.943	80.846
	1:320	67.096	61.935
FDM	1:80	1.251	0
	1:160	0	1.221
	1:320	1.114	0
AG	1:80	1.212	0
	1:160	1.231	1.212
	1:320	0	0
PAS	1:80	1.123	1.232
	1:160	0	1.253
	1:320	0	0
DTP	1:80	1.232	1.235
	1:160	1.321	1.123
	1:320	0	1.232
	1:80	0*	0*

*Mean of four Elisa values

Precision, LOD, LOQ

In order to survey the precision, the range between 55-850 EU/mL was calculated by the formula: (S1=850EU/mL, S3=214EU/mL, S5=55EU/mL). For the assessment of precision-repeatability, all OD values were calculated based on %CV and the values obtained as %CV≤ 14 in the intra-plate, %CV≤15 in inter-plate and %CV≤ 12 in the inter-day. All of the results fit the validation criteria which is mentioned in Table 2. To assess the Intermediate-precision, all of the OD values were also calculated based on %CV, with the values standing at Days %CV≤ 18 and Analyst %CV≤ 7, all of which fit the validation criteria. To determine the LOD, we followed the 2.12 paragraph which led to the assessment of 7 EU/mL. The assay of LOQ was conducted between 14-58 EU/mL (S5= 58EU/mL, S6= 27EU/mL, S7= 14EU/mL). All of the OD values were calculated based on %CV, showing

the value of %CV≤ 17 in all Inter-plate, Intra-plate and Intra-day precision, which fits the validation criteria. But, in Inter-day, the value of S7=14EU/mL %CV≤ 26 was obtained, which is not compatible with the criteria. Therefore, although the curve linearity in the 14-895 EU/mL is linear, the lowest amount of anti-PA4 which could be measured by ELISA method is shown to be 27 EU/mL.

Specificity

In Table-3, the increase of ELISA value was only observed in the Pool sample in all three dilutions of 1/80, 1/160, and 1/320 whilst other samples did not reveal any increase. So, the results of this table show that the anti-PA4 is completely specific and does not have any cross-reaction with other antigens. (Table 3)

Comparative analysis of anti-PA4 IgG

A competitive inhibition ELISA (CI-ELISA) was developed based on the qualified anti-PA4 ELISA. The aim of the CI-ELISA was to increase the specificity of the ELISA by reducing the incidence of false positives.

The PA4 was used to prove the ability of binding the antibody to antigen coated to the plate. This diagram shows that Anti-PA4 reacts with PA4 in a liquid-phase, and the PA4 antigen existing in the serum competes with the PA4 antigen coated in the plate on binding with the antibody; this leads to the reduction of ELISA value.

That means, the lower the PA4 in the serum, the more ELISA value will be, and the more it becomes, the less the ELISA value will get. The results of the study shown in Figure. 3 prove this claim.

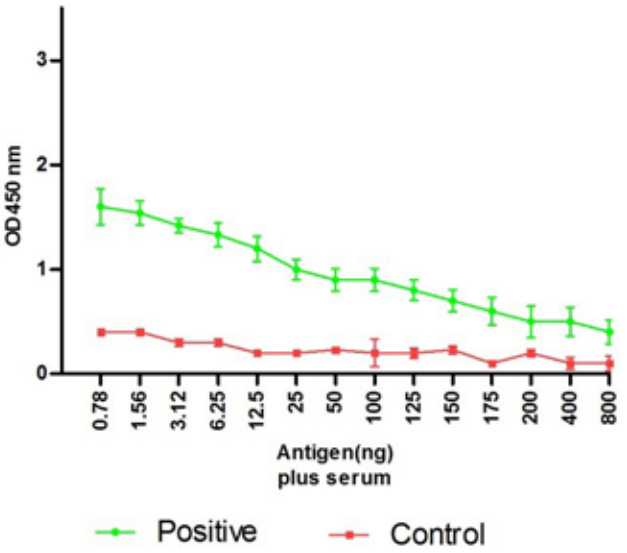

Fig. 3. A competitive inhibition ELISA (CI-ELISA). In the serum sample with the lower amount of PA4 antigen (0.78 ng) and the serum sample with the greater amount of PA4 antigen than the rest of the samples (800 ng), the highest ELISA value and the lowest ELISA value were observed respectively.

Comparing the amount of antibody against PA4 and LF in the vaccinated mice and the mice injected with virulent strain

We show that groups of AVA and Razi anthrax vaccine serums contain antibodies against the PA4 and LF but the Pool serum only has anti-PA4 whilst the NMS and *B. anthracis* 17JB groups lack such antibodies. Therefore, the results indicate that the mice having received the anthrax vaccine produce the antibody against the PA4 and LF after the third week (Table 4). Those injected with the strain 17JB only produce the detectible antibodies against LF after the fifth week. (Table 5),

Table 4. Comparing the amount of antibody against PA4 and LF in vaccine groups. A standard ELISA was performed for Anthrax vaccine adsorbed (AVA), Razi anthrax vaccine, Pool sera, NMS sera against anti PA4 and anti LF antigens. The ELISA value was significantly different between them.

Sample	Dilution in well	Elisa values	
		PA_4 coating	LF coating
AVA	1:80	25.403	21.37
Anthrax vaccine RAZI	1:80	28.225	24.193
Pool	1:80	97.983	0
NMS	1:80	0*	1.123*

*Mean of four Elisa values

Table 5. Comparing the amount of antibody against PA4 and LF in 17JB group. In order to detect antibody against PA4 and LF antigens, standard ELISA was performed for two groups of 17JB and NMS in the 3th and 5th weeks after the injection of strain 17JB. The Elisa value (after 5 weeks) was significantly different between them.

Sample	Dilution in well	Elisa value after 3 weeks		Elisa values after 5 weeks	
		PA_4 coating	LF coating	PA_4 coating	LF coating
17JB	1:80	0	1.209	1.151	17.195
NMS	1:80	0*	1.123*	1.021*	1.054*

*Mean of four Elisa values

DISCUSSION

The research conducted on the humoral immune response of the anthrax vaccines show that the role of anti-PA is the main factor of the vaccine's efficacy (Chen L, 2014). Therefore, future studies can look at this parameter more specifically to prove the immunity effects of the anthrax vaccine (Reuveny et al., 2001). The common ELISA kits have been monitored by full structure of PA. However, in an examination, the antibody measured is based solely on an important domain of PA (named PA4). The anti-PA4 can be an effective factor in the monitoring of the humoral immune response of all three existing vaccines against the *B. anthracis* (Flick-Smith et al., 2002).

To obtain the WHO and FDA license for manufacturing such vaccines for humans and animals, researchers need to survey the humoral immunity responses or anti-PA. In this study, we have developed an ELISA method detecting anti-PA4 (Weiss et al., 2007). All of the parameters have been assessed to validate the mentioned methods, and the results of the study show their validity for studying humoral immunity responses. As the study indicates, the coated PA4 antigen in the well plates did not cause any unspecific reaction against other livestock diseases as mentioned in Table 3. Moreover, through other validation tests, this test can be conducted in all laboratories by different researchers and bear the most accurate results. According to Table 4, using this method, the produced antibodies following the injection of anthrax vaccines in mice can be measured and the kinetic answers for future studies can be found. Although the most sensitive animal for such processes is guinea pig, using mice has several other advantages namely the convenience of testing and their abundance which make researchers forgo some of the disadvantages (Pombo et al., 2004).

One of the significant points in the application of this method, according to table 4 and 5, is that we created a model of anthrax disease in the mice via injecting the 17JB strain and measured the produced antibody against PA4 and LF, then compared it with the vaccinated groups. The positive control shows the increase in the antibody against PA4 and LF in the vaccinated groups despite what was shown in the disease groups.

Previous studies show that the amount of anti-PA in animals suffering from anthrax in the form of a skin disease has been increased. Therefore, the method, in which Anti-PA4 did not increase in comparison with the vaccinated group, can be a good option for screening cases suspected with infections from the vaccinated ones (Baillie LW et al., 2010; Ingram et al., 2010). The increase of anti-PA in the disease groups could be due to the presence of antibody against other PA's domains, which needs further studies. In addition, another reason for such diseases can be lack of PA in a pathogenic form (Ingram et al., 2010).

Although the performed studies represented that the levels of antibody increased against LF in the fifth week and this can be the point of difference between negative control and 17JB groups, the disadvantage of the described method is that it is not capable of distinguishing between the negative control group and the 17JB group during the first three weeks.

CONCLUSION

Through the validation tests carried out on this ELISA method, we showed that the detection range of the anti-PA4 can be 27- 892 EU/mL. We also believe that this ELISA method can be adopted in other laboratories in order to assess the immunity responses and differentiation of disease from vaccination after the first month. At the end, we can use this method as one of the alternative potency tests.

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CONFLICT OF INTEREST

None declared.

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