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IMMUNO-REACTIVITY AGAINST GIANTS VIRUSES BY ELISA SANDWICH TO DETECT ANTIBODY VERSUS ELISA USED NANOBODY TO DETECT ANTIGENS IN TUNISIAN PATIENTS WITH PNEUMONIA

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Abstract- Several studies have used serological tests to assess the prevalence of Mimivirus infections in patients with pneumonia. And better study the implication of these viruses during this pathology. The results observed by several teams were contradictory between those who support the idea of involvement of giant amoeba viruses in this pathology and those who predict the absence of involvement during this human disease. To report more evidence, we wanted to test the sera of patients hospitalized for this pathology extemporaneously. For this, we have developed a high-throughput semi-automatic ELISA system to test the reactivity of serum antibodies "ELISA Acs" but also for the first time an ELISA based on Nanobody "ELISA Vhh" which tests the reactivity of Vhh against particles or antigens of giant amoeba viruses belonging to the families Mimiviridae, Megaviridae, Marseilleviridae and Zamilon. The use of viruses of Tunisian origin aims to determine the relationship between the ecological niche and the specific pathogens because of the important polymorphism and the significant chimerization of its viruses which has not been studied in the various serological reports. or based solely on the Mimivirus prototype. Preliminary results show a reactivity of Tunisian sera with Tunisian viruses by ELISA Acs, but it was also higher with the LBA111 virus belonging to the Megaviridae family than with any other virus tested. ELISA Vhh tests were negative with all sera from blood donors and patients. But positive with synthetic serum from repeated dose injection of each virus in laboratory rabbits and harvest after 48h. The Elisa tests with all the synthetic sera of the other viruses were conclusive with a remarkable positivity which makes it possible to validate the Vhh constructs and also the antibody production reaction by the rabbits. We propose protocol to standardize the ELISA studies for its giant viruses and compare the results on a non-random basis by fixing the way of calculation of cut-off and thus give a mathematical approach for the determination of positive or negative serum. our purpose and to give a standard ELISA protocol for testing its viruses and we also propose Vhh constructs directed against a panel of its viruses for high flow tests of sputum, nasopharyngeal fluid, broncho-alveolar lavage, bronchic aspiration and sera for sale online via our portal. It was also noted that the reproducibility of the results and the number of false negatives were more conclusive with the use of Vhh than with ELISA Acs.
Key words: Mimiviridae, Megaviridae, Marseilleviridae, Zamilon virophage, ELISA, Nanobody, Pneumonia.

1. INTRODUCTION

The emergence of viruses in humans is not a new phenomenon. In the history of humanity, many viruses have appeared. Several human activities such as war, colonization and trade have led to the emergence of, for example, yellow fever and polio, which appeared suddenly in epidemic form, both in Europe and in North America. During the black slave trade, smallpox and measles were widely distributed in the Americas, striking non-immunologically affected Indian populations and causing the decline of pre-Columbian civilizations. But what scientists did not expect was the acceleration of this phenomenon in the second half of the twentieth century. Thus, viral hemorrhagic fever appeared in Africa and America, followed by AIDS (1981). New pandemics have emerged, such as H5N1 avian influenza (Hong Kong, 1997), H1N1 swine flu (1998 swine flu epidemic in the United States, human epidemic in 2009) or Chikungunya virus (southwestern China). SARS (South China, 2002), SARS-CoV (global epidemic 2003) NCoV or MersCoV (Middle East 2012), Ebola virus (Ebola outbreak in West Africa from 2013 to 2015) and Zika virus (Brazil 2015). These new viruses can occur unexpectedly, invade unknown regions of the world and sometimes settle permanently. In the face of these threats, our resources remain low: generally no vaccine, very few effective antivirals and sanitary measures that can be quickly exceeded. During the emergence or re-emergence of these viral diseases, man is the main culprit: since the Neolithic, he has appropriated the planet; it altered or destroyed many ecosystems by affecting the trophic chain while it was shaping others, entirely artificial and more exposed to the risk of emerging viruses. Giant amoeba viruses are among the new viruses that need their attention. The first isolation of a giant virus from a broncho-alveolar lavage, LBA111virus, revived the debate about the pathogenicity of its viruses. To explore this hypothesis, several studies using serological or molecular tests were conducted. Following the discovery of Mimivirus, sera from MIF-tested patients showed a statistically significantly higher prevalence of anti-Mimivirus antibodies

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in hospitalized patients in Canada than in controls [1]. In this work and another realize later, several pneumonia patients have seroconverted to Mimivirus [1, 2]. Since then, other serologic studies have shown higher Mimivirus seroprevalence in patients with acute lung disease, a significant association between anti-Mimivirus antibodies [2, 3]. Another published work using a multiplexed MIF study has shown that patients in the intensive care unit are commonly exposed to microorganisms associated with free amoeba [4]. The case of a laboratory technician in URMITE Marseille, France, also presented with unexplained pneumonia and concomitant seroconversion with 23 proteins of Mimivirus [5]. Finally, seroconversion has been demonstrated against antigens of Sputnik virophage in Laotian patients fed raw fish from the Mekong [6]. In contrast to these serological data, out of five reported molecular biology studies, only one revealed the detection DNA of Mimivirus [1]. The divergence between serological and molecular studies is thought to be due to the great genetic diversity of giant virus of amoeba despite cross-reactivity between isolates [7, 8, 9]. As the serological tests were carried out with the Mimivirus prototype strain, we decided to develop an ELISA to detect the reactivity of antibodies by ELISA Acs and antigens by ELISA Vhh for different giant Tunisian viruses. For the Marseilleviridae family, Tunisvirus; Mimiviridae family, Hirudovirus and Goulette virus; Megaviridae family, LBA111 virus, and Zamilon virophage. After recent clinical evidence of the isolation of LBA111virus from a broncoalveolar lavage associated with pneumonia in a Tunisian patient [10], we tested by ELISA a collection of Tunisian patients with pneumonia during their hospitalization. In this work, we also wanted to compare the reactivity of the serum antibodies detected by ELISA Acs to the reactivity of the serum antigen by ELISA Vhh.

2. RESULT

2. 1-Controls:

Immunofluorescence assay of LBA111 virus in blood donors:

We noted 90% of sera with a titer ≤1/ 50 and 10% with a titer 1/100 (Table 1). The best score of Sp and Se is calculating at dilution 1/100.

ELISA assay of LBA111 virus in blood donors with Cut off 0.8:

For the cut off 0.8 we have 6 positive sera which give us 100% sensitivity and 95% specificity see in Table 4. The test is validated we can apply its data to our sample series to target the reactivity of the sera of the patients.

Correlation between IF and ELISA assay:

Of the 9 sera of IF positive blood donors only 5 were ELISA positive. This allows us to say that the ELISA is more specific and more sensitive than the IF.

2.2-Patients:

After having determined the cut off and the background noise, the rule for the determination of positive sera is applied, this is as follows:

$$R = \frac{OD\ Sera\ of\ patient}{Cut-off} =$$

- Sera is positive reactivity≥ 1.4
- Sera is limit positive reactivity 1-1.4
- Sera is negative reactivity < 1

Or

$$R = \frac{Cut-off}{BN} =$$

- Sera is positive reactivity> 4
- Sera is limit positive reactivity
- Sera is negative reactivity < 4

Mimiviridae family:

For all the sera of patient tested against Hirudovirus the calculation does not validate serum with positive reactivity. For Goulette virus there are 4 serums that have a reactivity that exceeds the threshold of limit positive reactivity (between 1 and 1.125, see in Histogram 2).

Megaviridae family:

For all patient sera tested against LBA 111 virus the calculation validates 8 sera that have a reactivity that exceeds the threshold of limit positive reactivity (between 1 and 1.25, see in Histogram 2).

Marseilleviridae family:

Tunisvirus shows no positive reactivity with all patients' sera (see in Histogram 2).

Virophage Sputnik:

The reactivity calculations do not show any serum with a positive reactivity for Zamilon virophage (see in Histogram 2).

ELISA used Vhh to detect giant virus:
In this study we product the first typical Nanobody for each viruses cited. By comparison between the different values of Cut-off we retain the one that repeats (Cut-off=0.4 see in Table 3). The reactivity of sera blood donor and patients is negative for all tests, but positive with synthetic sera test (Histogram 3 and 4). All ELISA tests are done in extemporaneous to escape the problem of conservation of the serums for the serological tests of search antigens.

3. DISCUSSION

In this study, we demonstrated the strong correlation between the IF test and the ELISA test using blood donors (97%). This validates our internal ELISA test and the prototyped approach for a standard ELISA to explore the reactivity of sera from the Tunisian cohort to various Tunisian giant viruses. In this work the number of sera reacting with Goulette virus from Mimiviridae family, reaching the limit threshold of positive reactivity is equal to 5 sera. It may be noted that the closest strain of Mimivirus in this study is Hirudovirus does not give any positive reactivity with any patient serum that could explain that the choice of this prototype in serological studies was very random and based on the fact that it is the first strain discovered in addition to the link with pneumonia lack of scientific evidence despite all the evidence already proposed. Indeed, several arguments are proposed namely Ghigo E et al have demonstrated that Mimivirus is internalized by macrophages [16]. The pathogenicity of Mimivirus tested on a mouse experimental model suggesting a particular tropism of this virus for the lungs [17]. A sero-epidemiologic study of 376 Canadian community-acquired pneumonia patients compared to a group of 511 healthy subjects showed that pneumonia patients had significantly higher levels of anti-Mimivirus antibodies than patients with pneumonia. those in the control group (9.66% vs. 2.3%, respectively) [1]. A second study, carried out in intensive care unit in patients with nosocomial pneumonia, also showed the existence of a correlation between pneumonia and the presence of anti-Mimivirus antibodies [2]. Positive Mimivirus serology was detected in 59 patients with pneumonia [3]. A study of the Horn chronic respiratory reference center in the Netherlands showed that 3 serum samples from three different patients had anti-Mimivirus antibodies (2.5%) [18]. The case of the laboratory technician who developed subacute pneumonia with seroconversion to Mimivirus [22]. Several molecular biology studies (PCR) of respiratory specimens or related to this pathology have highlighted this virus [1, 10, 19]. A Metagenomic study has shown the existence of two Mimivirus sequences on samples of nasopharyngeal aspirations of patients with severe pneumonia [21]. Human isolates such as LBA111 virus in BAL or Shanvirus in stool of a Tunisian patient with pneumonia [10, 19]. Contrary to his studies, our cohort does not plead for a tropism or a possible implication of the strain Mimivirividae family, Hirudovirus, in this pathology. Our study is associated with other studies which does not evoke the certain role of Mimivirus during this pathology. Although the number of serum positive against Goulette virus also suggests questions about the possibility that other distant phylogenetic strains of Mimivirus could explain this reactivity. The ELISA tests with the Vhh-Hirudovirus and Vhh-Goulette virus were negative, which further suggests that the same sera show serum reactivity when the virus or an epitope of it is not found. This work also shows that Marseilleviridae family in our case Tunisvirus have a low reactivity, indeed no serum has reached the limit threshold of positive reactivity with ELISA Acs or with Vhh-Tunisvirus. This suggests that his viruses have no role in this pathology. This seems compatible with the literature review or the only strain belonging to Marseilleviridae family called Senegal Virus was isolated from the stool of a Senegalese man in good health [23]. Also the genome of another virus of the same family called GBM for "Giant Blood Marseillevirus" was found in the blood of asymptomatic donors [24]. For the LBA111 virus, the number of sera that reached the positive reactivity threshold was 8 and this is the highest number requiring further reflection on this reactivity. It should also be noted that extemporaneous ELISA tests against Vhh-LBA111virus were negative whereas it was positive with synthetic sera. Despite this presence of positive reactive sera with LBA 111virus which is an isolated strain in case of pneumonia, we cannot establish a direct link with the disease due to the absence of other reported cases. In the case of the Zamilon virophage, no serum reached the threshold of positive reactivity with both ELISA techniques. We can assume that reactivity depends on ecological niche and antigenic homology. It should also be noted that the difference in reactivity between Mimiviridae and Megaviridae can be explained by the size, the number of fibrils and the presence of a fibrillic structure called "Cowlicks" typical of Megavirus (LBA111 virus). The product Vhh allows a better serological study because of the great specificity, the size of Nanobody so that the reaction is present at a sufficient threshold or absent it was demonstrated by the absence of positive serums of blood donors with the test ELISA Vhh while the test were positive with synthetic sera. In this study, we had positive limit reactivity with the blood donors to the number of 5 sera close to that detected for the sera of patients with ELISA Acs but with no blood donor serum for ELISA Vhh. This shows a better specificity and sensitivity of the ELISA Vhh technique, but also underlines that LBA111virus already detected in a broncho-alveolar lavage may be a contamination due to a manipulation error, notably that its viruses are deemed ubiquitous in all environments. This work concerns only a small cohort of patients in whom it can be said that giant amoeba viruses are not involved in pneumonia but that their detection during this disease is due to their important presence in all surround us. To better understand this presence of LBA111virus in the broncho-alveolar lavage 111 [10], we conducted a study at the Rabta Hospital in different departments, in particular the department of pneumology, in which the strain LBA111virus was discovered to trace the history and the links between its viruses and the environment and see if it is not a contamination of the sample during handling. This work in progress will be published shortly. In this work, we have been able to propose a standard ELISA method based on the search for antibodies but also to show the added value of the Vhh-anti giant virus of amoeba that we propose for sale via our online portal.

4. MATERIALS AND METHODS

Patients and methods

4.1 Patients

Study procedures were approved by the Ethical Review Committee (RABTA TUNISIA 0015). Written (signature or thumbprint) informed consent was obtained from all adults participants, supported by the signature or thumbprint of a second adult witness to the consent process; parental consent was sought for children, accompanied by age-appropriate assent. We collected information on the main demographic variables (age, sex, and occupation), time from symptom onset to hospital admission, co-existence of chronic diseases (chronic pulmonary diseases, diabetes, renal diseases, chronic cardiac disease, asthma or tuberculosis, Tabagism, Neffa, Tabouna status (yes or no) in occurrence of pneumonia. Sera samples were collected from 116 Tunisian patients with community acquired pneumonia. Diagnosis of pneumonia was considered when a pulmonary infiltrate was present on a chest radiograph in combination with at least two of the following symptoms: cough, sputum production, a temperature > 38 °C, auscultatory findings consistent with pneumonia, a C-reactive protein concentration >15 mg/l, and a white blood cell count >12×109 cells/l or < 4×109 cells/l (Fine MJ, Am J Med, 1993). We used 99 of anonymous blood donor collected from Tunisian healthy patients without history and pneumonia infection were randomly selected to be used as controls and the synthetic sera after virus inoculation in laboratory rabbits we recover the serums after inoculation at different intervals this will allow to have the positive sera for our tests Elisa. All sera samples were pelleted by centrifugation at 500g for 10 min and supernatant was kept and then stored at -20°C to process for ELISA assay.

4.1.1 - Origin of antigens:

We used as antigens, 5 Tunisian giants viruses and one virophage: Hirudovirus[11], Goulette virus [12], LBA111 virus [10], Tunisvirus [12] and Zamilon virophage [12, 13]. These viruses came from different ecological niches and patient with pneumonia in Tunisia (Published data).

4.1.2 - Production of antigens:

Antigen production was performed as previously described [14]. Briefly, production starts by inoculating 500µl of each virus in 5 ml of Acanthamoeba spp rinsed in sterile Page's amoebal saline (PAS) into a cell culture flask 25 cm2 (Sigma Aldrich, Saint-Quentin Fallavier, France). The absence of bacterial contamination was verified by inoculation of a drop of each virus suspension on BCYE agar, Colombia sheep blood agar, and by Gram and Gimenez staining. After complete lysis (2 to 3 days), unlysed amebas were removed by low speed centrifugation at 2000 rpm for 10 min. Purification of antigens consists in centrifuging, the total volume of each virus produced at 10,000 rpm for 20 min for Hirudovirus, Goulette virus, Tunisvirus and LBA111 virus and at 22,000 rpm for one hour for Zamilon virophage. Each pellet of virus was washed three times with sterile Phosphate-buffered saline (PBS), and then resuspended in 2ml of PBS sterile buffer. The protein concentration was determined using a Bradford assay (Bradford, 1976).

4.1.3 - Production of Vhh:

Nanobody production was performed as previously described by Pardon E et al [15]. Briefly, as for the production already described for antigens. Once the viral solutions are ready, 1 ml of the final viral solution is injected into the camels for immunization. We Mix 6 antigens (each stored at 100 mg/mL). Just before immunizing, 1 aliquot was used to infect amoeba. After lysis we use 2ml of virus solution purified for each virus. Thaw one aliquot (2ml for each virus solution) and combine with an equal volume of adjuvant, mix well, and inject subcutaneously in a camelid. Inject a maximum of 2 mL of the mixture per spot subcutaneously and inject at up to five spots. Repeat this immunization every week for five times. The following steps are described previously [15].

4.1.4- Immunofluorescence assay:

The blood donors used as controls negative in our study were tested against LBA111 virus by Immunofluorescence assay and synthetic sera with LBA111 virus used as control positive. The concentration of the virus in the suspension was 109/mL of PBS and the sera were diluted at 1:50 to 1:400.

4.1.5- ELISA assay

ELISA plates Nunc Maxisorp were coated with 0.4 µg/well of each virus extract in 100 µl per well of carbonate buffer (0.05 M, pH 9.6) overnight at 4°C. Unbound antigens were removed by washing using a plate washer and PBS buffer containing 0.1% Tween (PBST) for three times. The plate was blocked for 30 min at room temperature, after addition of 200 µl PBS buffer with5% milk powder in each well. Then the plates were washed PBST buffer three times. The serum samples were diluted to 1:1000 in PBS buffer with 3% dried milk and 0.1% Tween. Then, 100µl of diluted samples were added to each well and were incubated for 1 h at room temperature. The plate was washed with PBST buffer at 3 times and incubated with 1:10.000 Anti-Human IgG coupled with Alkaline Phosphatase as a secondary antibody (Sigma Aldrich, Saint-Quentin Fallavier, France) in PBST buffer containing 3% milk with gentle shaking for 1 hour. After three washes PBST buffer, 200 µl

of Para-Nitrophenyl Phosphate (Sigma Aldrich, Saint-Quentin Fallavier, France) was added to each well for 30 min. The absorbance value was measured at 405 nm using a microtiter plate reader (Multiskan FC). For the ELISA with Vhh, the optimal Vhh for each virus was determined using viral solutions of several concentrations from 101 to 105 in the solutions prepared with PBSTween 0.1%, 3% and then with serum already tested negative with different viruses but positive with synthetic sera. The Vhh select are used to detect the presence of viral antigen in patients coupled with alkaline phosphatase. All the sera (patients and blood donors) were tested in duplicate. Two blank controls containing an anti human-IgG coupled to phosphatase alkaline (1:10.000) (Sigma Aldrich, Saint-Quentin Fallavier, France) in PBSTween 0.1%, 3% skimmed milk, in place of the negative control standard, were submitted to the same protocol to control the background noise (BN).

4.1.6- Analysis of results of IF blood donors

For the different dilutions used (0, 1/50, 1/100, 1/200, 1/400) the sensitivity (Se) and the specificity (Sp) of the test were calculated on the basis of the results in Table 1. The details of the calculation matrices are given in Table 2.

4.1.7- Analysis of results of ELISA blood donors

For the sera of blood donors, a mean value with a standard error was calculated. For sera from blood donors the OD with background noise (OD=0.209) was measured (see Histogram 1). We apply several formulas of calculation of Cut off and comparing with the real situation of the blood donors we notice that three among the formulas quoted in Table 3 gives us the same values of 0.8 one other are close to this values. Thanks to this comparison we could define in a non-arbitrary way the Cut off equal to 0.8 see Table 3.

Table 1: Classification number of sera positive of the dilution antigen LBA111 virus for blood donors

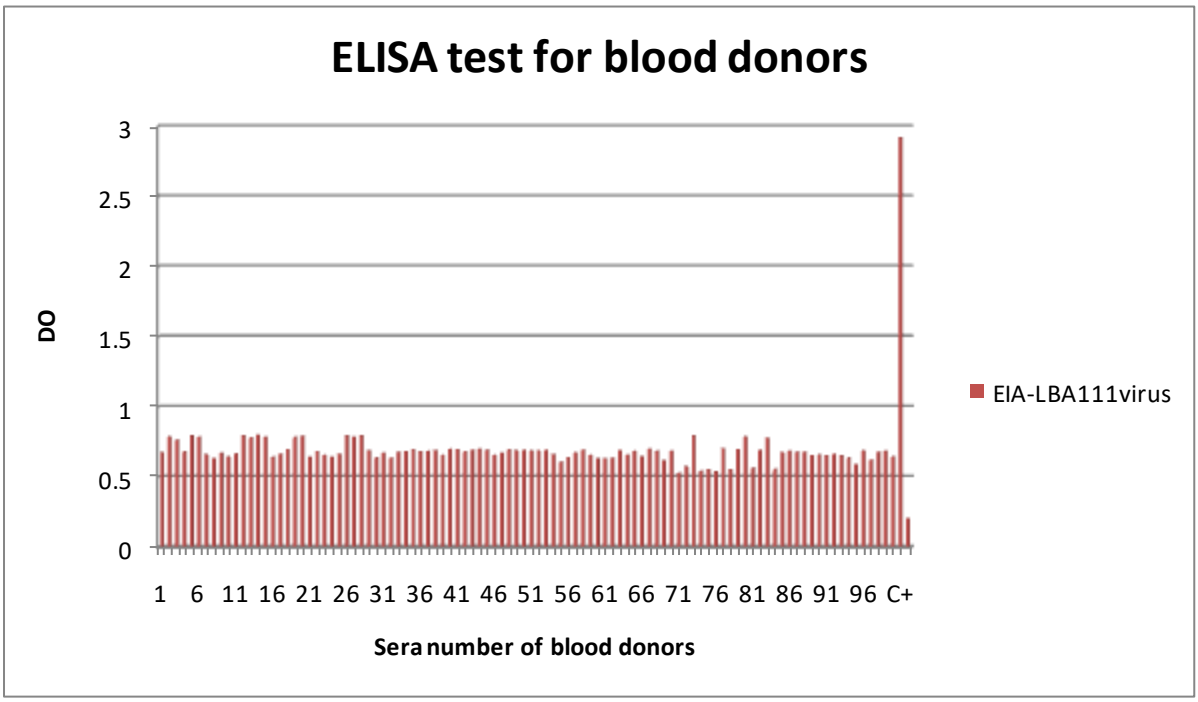
Dilution	0	1/ 50	1/ 100	1/ 200	1/400
IF	80	26	18	0	0

Table 2: Calculates specificity and sensitivity according to relationships of pneumonia disease status and IF test dilution

	True disease state	
Test	D+	D-
T+	TP (true positive) 1 (for all dilutions)	FP (false positive) 79 (dilution 0) 25 (dilution 1/50) 17 (dilution 1/100) 0 (dilution 1/200 or 1/400)
T-	FN (false negative) 0 (for all dilution)	TN (true negative) 99 (for all dilutions)

Diagnostic (Clinical) sensitivity (Se) = [TP/ (TP+FN)] x 100
Diagnostic (Clinical) specificity (Sp) = [TN/ (TN+FP)] x 100
Efficiency (Ef) = [(TP+TN)/(TP+FP+TN+FN)]
Youden Index (J) = Se+Sp-1
Likelhood Ratio (LR+) = Se/1-Sp
Likelhood Ratio (LR-) = (1-Se)/Sp
[(Se (0) =100%; Sp (0) = 55%), (Se (1/50) =100%; Sp (1/50) = 79%), (Se (1/100) =100%; Sp (1/100) = 85%), (Se (1/200 or 1/400) = 50%; Sp (1/200 or 1/400) = 100%)].
Ef= 0.85
J= 0.85
LR+= 6.66
LR-= 0

Histogram 1: DO measurement for blood donors against LBA111virus by ELISA Acs



The standard deviation = 0.07; Median = 0.67; Background noise = 0.209.

Table 3: Calculates Cut-off formulas

Formula	a	Computation	Cut-off	
			ELISA Acs	ELISA Vhh
1	2	2 x MEAN of negative controls f =0	1.3	0.4
2	3	3 x MEAN of negative controls f=0	1.9	0.6
3	1	MEAN of negative controls + 0.13 x MEAN of positive controls Cutoff = $\bar{A}$ neg + 0.13 $\bar{A}$ pos f=0 Pan et al. (1992) formula [26]	1.1	0.7
4	1	MEAN + f x SD, with f = 2.079 Cutoff = a. $\bar{A}$ + f . SD f = t.√1 + (1/j) Frey et al. (1998)[27]	0.8	0.4
5	1	MEAN + f x SD, with f = 3.452 Cutoff = a. $\bar{A}$ + f . SD f = t.√1 + (1/j) Frey et al. (1998)[27]	0.9	0.5
6	1	(MEAN + 3 x SD) of negative controls $\text{Cutoff} = \bar{X} + \text{SD}\sqrt{1 + (1/n)}$ $\text{SD} = \sqrt{\frac{\Sigma(x_i - \bar{X})^2}{n-1}}$ f=3 Classen et al. (1987)[28]	0.8	0.4
7	1	(MEAN + 2 x SD) of negative controls	0.8	0.3

Table of Frédéric Lardeux et al modified [25]: For the computation of F4 and F5, j is the number of negative controls used in the plate (100 in the present study) and t is the (1-α)th percentile of the one-tailed Student t-distribution with (j-1) degrees of freedom. Because 100 negative controls were used in the study (separate in groups of 30 and mathematically adapt by addition of results and average search to be able to apply the rule of Frey et al 1998, we apply the formula mentioned below *), and taking into account the confidence level for the computation of the Student t, the f values were 2.079 Confidence level (1-α) for t computation: 97.5% and 3.452 Confidence level (1-α) for t computation: 99.9 % for F4 and F5 respectively.

*For the F4 and F5 we apply the following formula: $\sum_{n=1}^{100}(x) = \sum_{n=1}^{30}(x) + \sum_{31}^{60}(x) + \sum_{61}^{90}(x) + \sum_{91}^{100}(x)$

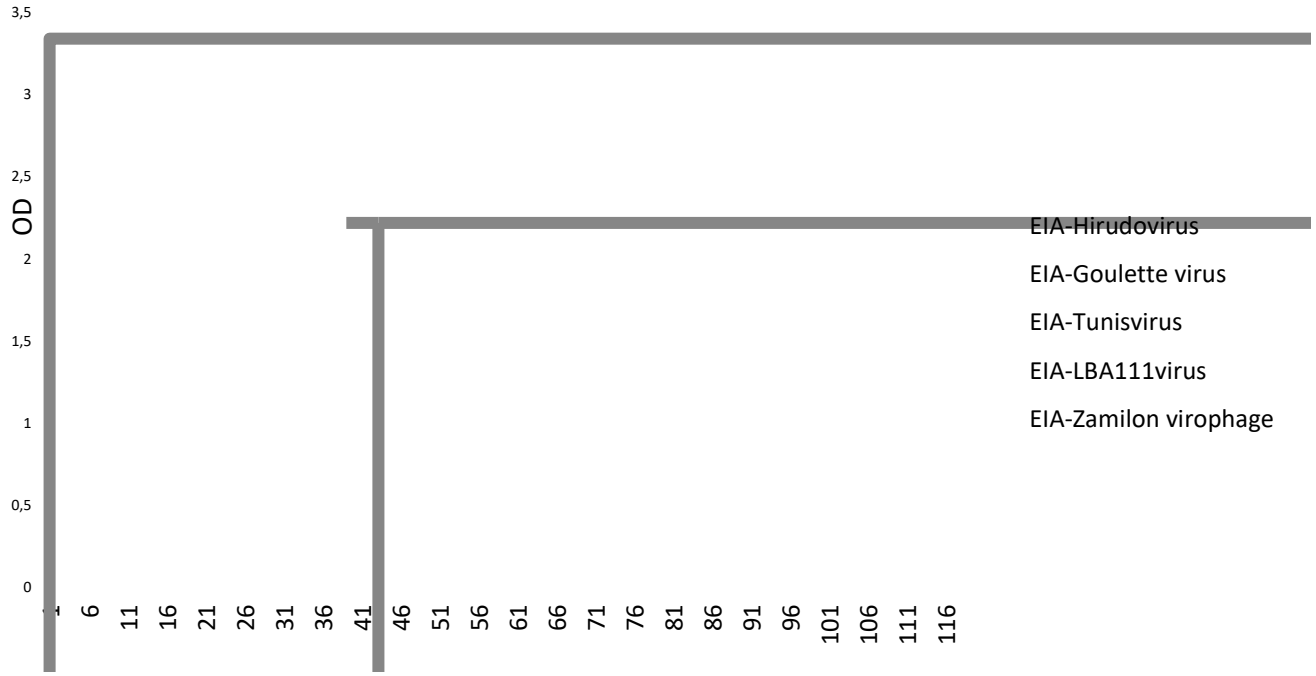
$$X = a.\ \bar{A} + f.\ SD$$

Table 4: Calculates specificity and sensitivity according to relationships of pneumonia disease status and ELISA test (Cutoff = 0.8)

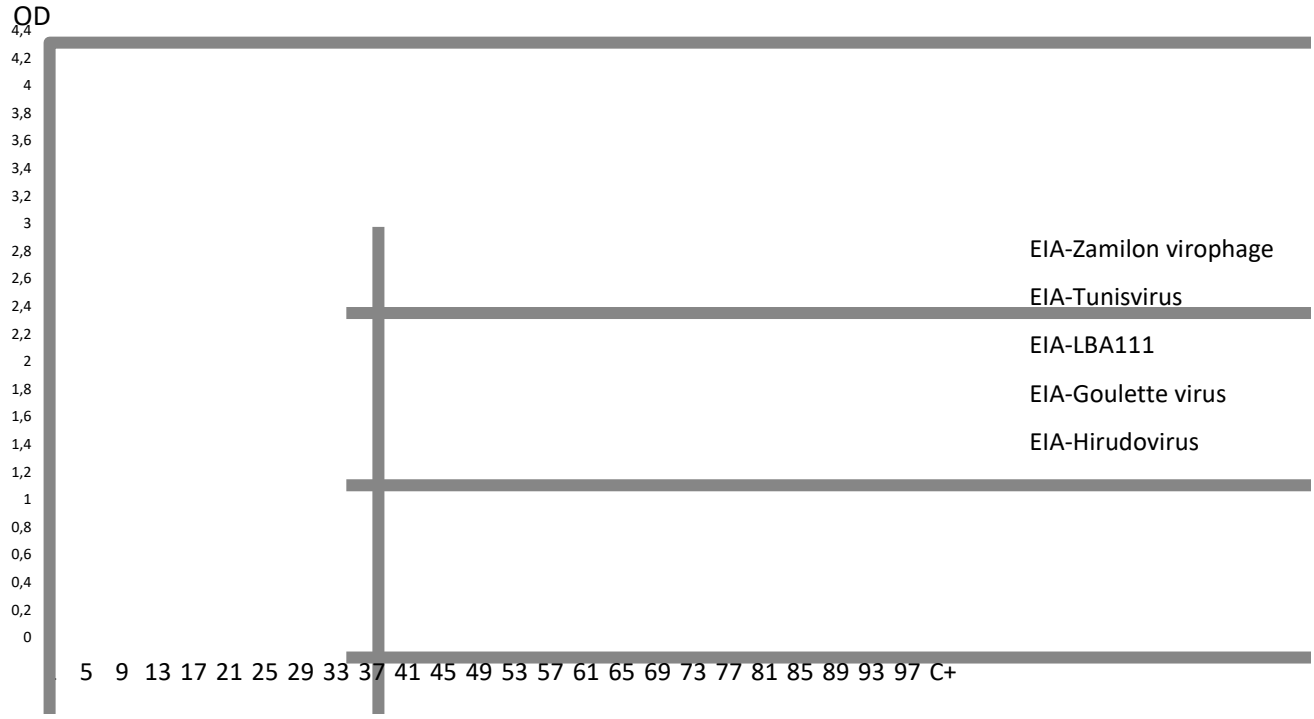
	True disease state	
Test	D+	D-
T+	TP (true positive) 1	FP (false positive) 5 (Cutoff 0.8)
T-	FN (false negative) 0 (for all dilution)	TN (true negative) 99 (for all dilutions)

Diagnostic (Clinical) sensitivity (Se) = [TP/ (TP+FN)] x 100
Diagnostic (Clinical) specificity (Sp) = [TN/ (TN+FP)] x 100
Efficiency (Ef) = [(TP+TN)/(TP+FP+TN+FN)]
Youden Index (J) = Se+Sp-1; Likelihood Ratio (LR+) = Se/1-Sp; Likelihood Ratio (LR-) = (1-Se)/Sp
[(Se (0.8) =100%; Sp (0.8) = 95%]; Ef=0.95; J=0.95; LR+=2; LR- =0.

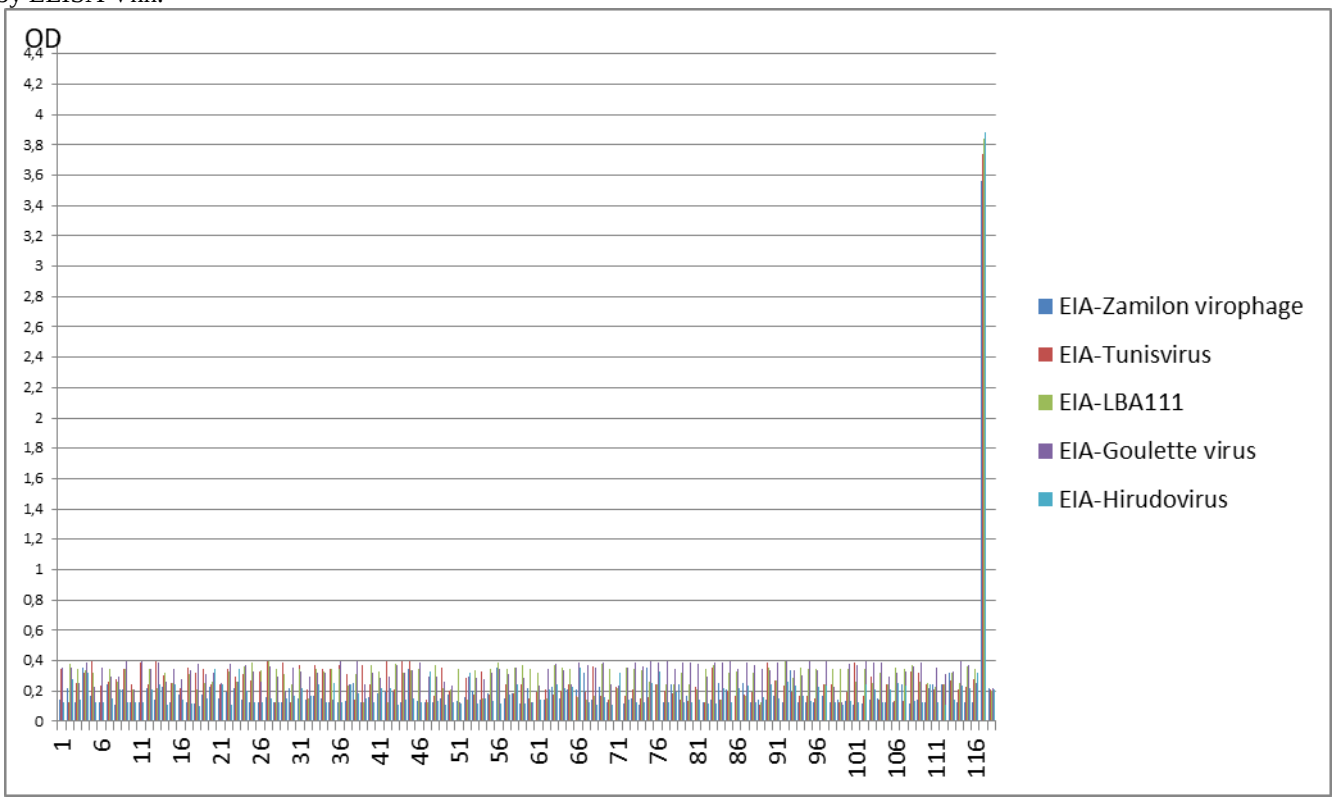
Histograms 2: DO measurements for sera patient against Mimiviridae, Megaviridae Marseilleviridae and Zamilon virophage by ELISA Acs.



Histograms 3: DO measurements for sera blood donors against Mimiviridae, Megaviridae Marseilleviridae and Zamilon virophage by ELISA Vhh.



Histograms 4: DO measurements for sera patient against Mimiviridae, Megaviridae Marseilleviridae and Zamilon virophage by ELISA Vhh.



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