Optimization Of An Elisa Assay For Detecting Hiv-I Mper Antibodies In HIV-I Discordant Couples

By

Chukwuma George Okechukwu[1] , Ahaneku Gladys Ifesinachi[2], Agbakoba Nneka Regina[1], Enweani Ifeoma Bessie[1], Chukwuma Oluchukwu Mariagoretti[1], Manafa Patrick Onochie[1], Loveline N. Ngu[3], Apeh A. Ngoh[3], Abel Lissom[3], Herve F. Ouambo[3], Doline, Takoua[3], Suzanne H. Magagoum[3], Thibau F. Tchouangueu[3], Arinze S. Okoli [4], , Alain B. Waffo[5], Esimone Charles Okechukwu[6], Nchinda Godwin W.[3]

[1]Department of Medical Laboratory Science, Faculty of Health Sciences & Technology Nnamdi Azikiwe University, Awka

[2] Department of Medicine, Faculty of Medicine, Nnamdi Azikiwe University Awka

[3] Laboratory of Vaccinology/Biobanking, CIRCB Cameroon

[4] GenØk - Centre for Biosafety, Tromsø, Norway

[5] Microbiology Program, Department of Biological Sciences, College of Science, Technology, Engineering and Mathematics, Alabama State University, Montgomery, AL, USA

[6] Department of Pharmaceutical Microbiology & Biotechnology, Faculty Of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka

Corresponding Author
Dr Chukwuma George Okechukwu
Department of Medical Laboratory Science
Faculty of Health Sciences & Technology
Nnamdi Azikiwe University Awka
+2348034101608
go.chukwuma@unizik.edu.ng

Abstract

ELISA techniques have been of great use in antigen and antibody detection. In this study, we have optimized an in-house indirect ELISA technique for antigenicity assays involving HIV-I discordant couples. We investigated the ability of the HIV-I QBMPERN protein to be used in detecting antibody responses in HIV-I seropositive and highly exposed but seronegative individuals. This study was aimed at developing and enhancing an Indirect ELISA Assay technique. Our group had engineered the hybrid phage HIV-I QBMPERN. Optimal requirements for ELISA assays, which include the concentration of the coating antigen, labeled antibody concentrations, temperatures and incubation times were determined. To confirm the efficacy of our assay technique, ethical approval was obtained, informed consent sought and questionnaire were administered. Serum samples were collected from 24 HIV-I discordant couples, analyzed and results were read using Thermofisher multiscan ELISA reader. The 31-40 years age bracket had the

most infection HIV-I infection while the 51-60 years age bracket were least infected by HIV-I. Older men had more HIV-I infection than older women while younger women were more infected than younger men. Forty two percent of men and 58% of women were positive for HIV-I among the discordant couples. Optimal conditions were considered in performing the ELISA assay. The IgG antibody response obtained in this study was strong for both the HIV-I seropositive and seronegative couples. This study has confirmed the efficacy of our optimized in-house ELISA assay at detecting antibody responses associated with the HIV-I MPER. The assay was found to be efficacious and cost effective.

Key words: HIV-I QBMPERN, ELISA, discordant, HIV-I, antibody response

INTRODUCTION

Broadly neutralizing antibodies (bNAbs) which targets some regions within the human immunodeficiency virus type-1 (HIV-1) envelope glycoprotein (Env) can be produced by the immune system of humans. These antibodies can be induced by vaccination and this helps to protect against a wide array of viral diversity. We have used recent innovations (Singleton et al, 2014; Waffo et al, 2014) in our group to produce novel immunogens which target conserved bnAb epitopes in the HIV-1 gp41 membrane proximal external region (MPER). We have used the HIV-1 gp41 MPER because it is one of the most conserved regions of Env which plays significant roles in viral infectivity and fusion. Moreover, the MPER region is as well targeted by various bnAb including 2F5, 4E10, Z13e1, m66, m66.6, 10E8 and CAP206-CH12 (Buchacher et al, 1994; Muster et al, 1993; Nelson et al, 2007; Ofek et al, 2014, Zhu et al, 2011; Zwick et al, 2001; Huang et al, 2012; Morris et al, 2011). The bnAb of the 10E8 origin are amongst the broadest and most dominant bnAb reported. The evolution of broadly neutralizing antibodies (bNAbs) against HIV has been a success in the past few years as many bNAbs were assessed in clinical trials These clinical trials were restricted to individuals living in the United States and Europe, thereby reducing the evaluation of the global utility of these antibodies, this is because most of the residents in the regions in question were infected with clade B HIV-1 (Zhu et al, 2011). In a bid to determine the antigenicity of the MPER antigen developed by our consortium for possible diagnostic and vaccine efficacy, we optimized an ELISA assay for the evaluation of HIV-I QβMPERN protein associated broadly neutralizing antibodies from HIV-I infected and HIV-I uninfected but highly exposed individuals. Consequently, blood samples were collected from HIV-I discordant couples in Southeast Nigeria for antigenicity studies. The antibody titers gotten with our assay showed strong responses. An evaluation of different assays, which includes PCR, loop -mediated isothermal amplification (LAMP), radio immune assay (RIA) and ELISA, showed that ELISA has several benefits (Hu et al, 2008; Riera et al, 1985; Torigoe et al, 2007; Kaplan et al, 1983). In- house ELISA assays are required because of their low cost and convenience. In addition, using commercial ELISA kits to detect antibody response, especially for vaccine related studies could cost much more than in-house kits. It was therefore important that we developed and

optimized an indirect-ELISA assay for the detection of MPER specific antibodies

Materials and Methods

Study area

The study area was Awka in Southeast region of Nigeria. Awka is the capital city of Anambra State. It was declared capital on 21 August 1991, after the creation of Anambra and Enugu states. The capital was moved from Enugu to Awka. The city has an estimated population of 301,657 as of the 2006 Nigerian census, and over 2.5 million as of a 2018 estimate. Awka is located at 199.1 kilometres (123.7 mi), by road, directly north of Port Harcourt in the heart of the densely-populated Igbo land in South East Nigeria. The West-East Federal highway links Lagos, Benin City, Asaba, Onitsha, and Enugu to Awka and many other local road networks link it to other towns such as Oko ,Ekwulobia, Agulu, Enugwu-Ukwu, Abagana and Nnewi. Awka is located midway between two major cities in Northern Igboland, Onitsha and Enugu, and this was considered in its choice as an administrative center for the colonial government and today as a platform for the Anambra State government.

Study site

The study was performed at two centers which include Anambra State University Teaching Hospital Awka and the Chantal Biya International reference centre for research on the treatment and management of HIV/AIDS (CIRCB) Yaounde, Cameroon. At the Awka center, samples were collected from HIV-I discordant samples using convenience sampling technique (it should be noted that the subjects came from various towns within the Southeast region of Nigeria). Samples were collected from HIV-I infected individuals as well as their seronegative partners. The samples were processed and then stored at -80^{o}c before being transported to CIRCB, Yaounde Cameroun. As the Capital city of Cameroon Yaounde (3°52′N11°31′E) is a multi-146 ethnic city located at an average height of 750 m. Yaounde, is situated in the centre of the rain forest. The temperature is around 23.7°C. The CIRCB is well equipped for vaccine research and is located around the Mellen area of the city

Study design

This cross sectional study was conducted among HIV-I discordant couples. Subjects were selected using convenience sampling technique. Individuals with other infections (including microfilaria, dengue, TB, and hepatitis B and C) and pregnant women were not included in the study.

Study population

The study population consisted of 24 consenting discordant couples conveniently selected from the HIV-I clinic of the Anambra State University Teaching Hospital Awka. Of the 48 participants in this study, 24 were males while 24 were females.

Ethical approval

Ethical approval for this study was gotten from the ethics committee of the Anambra State University Teaching Hospital Awka, Anambra State Nigeria **(COOUTH/AA/VOl.X1/039)**

Sample collection, transportation and processing

About 5 mls of blood was collected into plastic Vacuum K2EDTA container called Vacutest (Vacutestkirma, Italy). Samples were collected between February and March, 2017 All samples were stored at room temperature and processed within 4 hours of collection. Plasma was obtained by centrifuging samples at 2,000 rpm for 10 min at 4°C. The plasma fraction was harvested sterile under the hood, aliquoted and stored in duplicates at -20°C until use. Subsequently, samples were transported to the Vaccinology laboratory of Chantal BIYA International Reference Centre (CIRCB) Yaounde Cameroun for storage and analysis. The plasma obtained from participants was heat inactivated for 30 minutes at 56°C prior to ELISA assay.

Study antigens

The recombinant phage QβMPERN was used as the antigen for this study. In previous experiments, it showed strong reactivity with Z13e1, 4E10 and the strongest reactivity with 10E8 and as such was selected for this study

HIV screening

The Geenius confirmatory assay device was labeled with the patient ID or identification number. Using a laboratory pipette, about 5 µl of serum was added to the centre of the SAMPLE + BUFFER Well 1 of the device. Immediately following the addition of the sample, the diluent dropper bottle was used to add 2 drops (60 µl) of Buffer into the SAMPLE + BUFFER Well 1 and allowed for 5-7 minutes. The diluent dropper bottle was used to add 5 drops (150 µl) of Buffer into BUFFER Well 2. The result was read between 20 to 30 minutes after the addition of the Buffer to BUFFER Well (manufacturer's instructions)

Determination of antibody responses using an in-house ELISA technique

ELISA plates (Thermos Fisher Scientific, USA) were coated overnight with 10^7 pfu/well of the recombinant phage QβMPERN. Plates were washed 4x using PBST (PBS containing 0.05% Tween 20) and was blocked for one hour with 3% BSA in PBST at 37º C. Heat inactivated plasma from HIV-1 infected participants and their seronegative partners (ie discordant couples) was diluted 1:200 in PBS then added in each well (100 µl) of the antigen coated plates and incubation was performed for two hours at 37º C. The plates were washed 5x (98 µl/well) with PBST and subsequently, the bound antibody was probed with a peroxidase-conjugated mouse anti-human IgG (Southern Biotech) diluted 1:2000 in PBS. Bound conjugate was confirmed using ABTS substrate and stop solution according to the manufacturer's instructions (southern biotech,). The colorimetric signal was measured at 405 nm using a multiscan FC microplate reader (Thermo Fisher Scientific, USA).

Statistical analysis

Data analysis was carried out using Graphpad Prism Software version 6.1. Data were expressed as median (25th percentile-75th percentile). Comparisons of medians among two groups were performed by the U-Mann-Whitney test. Statistical significance was confirmed when $P < 0.05$.

RESULTS

Table 1: Distribution of HIV-1 discordant couples according to age

In this study, we included 48 HIV-I discordant couples selected from the HIV-I clinic of the Anambra state university teaching hospital Awka, Nigeria. Twenty four of the participants were HIV-I seropositive while their seronegative partners were also 24 in number. The ages of the study participants and their HIV-I status is shown. The 31-40 years age bracket had the most infection while the 51-60 years age bracket were least infected. Older men had more HIV-I infection than older women while younger women were more infected than younger men. Married men were more seronegative while married women were more seropositive to HIV-I infection.

Table 1: Distribution of HIV-1 discordant couples according to age

Age group	Male:n=24		Female: n=24	
	HIV –I negative	HIV-1positive	HIV-I negative	HIV-1positive
21-30 n=8(16.6%)	0(0%)	0(0%)	4(16.65%)	4(16.65%)
31-40 n=25(52.1%)	7(29.2%)	5(20.8%)	6(25%)	7(29.2%)
41-50 n=13(27.1%)	6(25%)	4(16.6)	0(0%)	3(12.5%)
51-60 n=2(4.2%)	1(4.2%)	1(4.2%)	0(0%)	0(0%)

Figure 1: Distribution of HIV-I infection in discordant couples

The pattern of HIV-I infcetion among discordant couples shows that a greater percentage of females (58%) were infected than males (42%). A greater percentahe of males were seronegative. There was no significant relationship between males and

females for both the infected and uninfected couples.

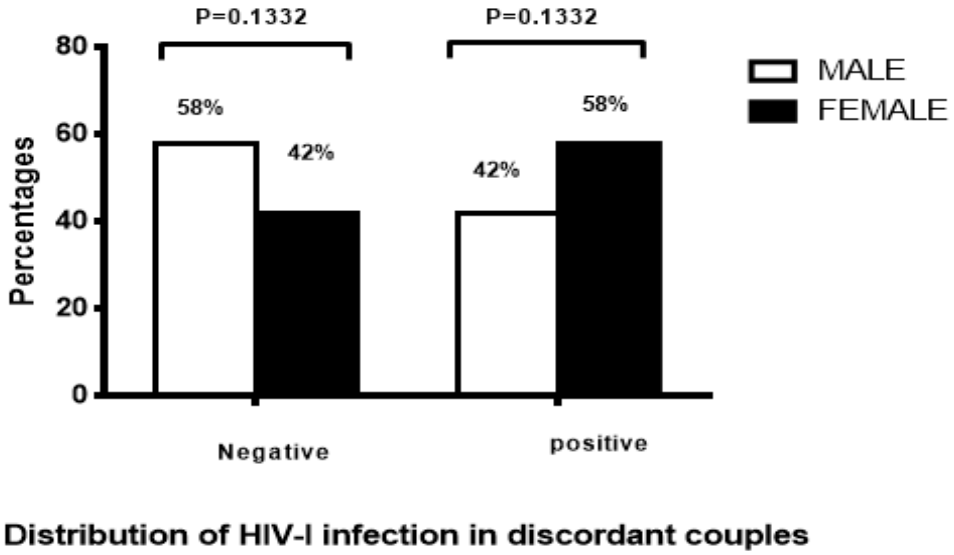

Figure 1: Distribution of HIV-I infection in discordant couples.

Figure 2: IgG antibody responses among HIV-I discordant couples

Figures 2 shows scatter plots for IgG antibody responses. The dots represent responses from the discordant couples and each indicates the size of response associated with broadly neutralising antibodies specific for HIV-I QβMPERN. For the IgG antibody response, there is no significant difference between the immune response of the infected and non-infected couples (p=0.1334). The seronegative couples seem to have slightly weaker responses than their seropositive partners, even though both shows considerably strong antiboby responses.. The midline indicates the median, The immune response pattern of the seronegatives are more dispersed despite having higher OD.

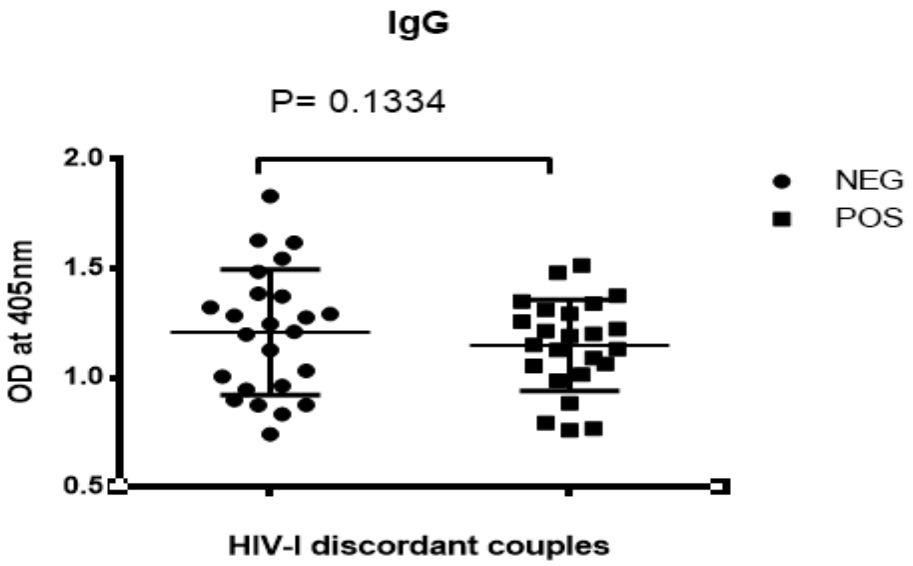

Figure 2: IgG antibody responses among HIV-I discordant couples

DISCUSSION

This study aws aimed at developing and optimizing an in-house ELISA assay for detecting MPER specific antibodies in HIV-I discordant couples. For this purpose, we used a HIV-I QBMPERN engineered by our research team. The gp41 MPER is highly conserved and rich in bnAb epitopes, it plays key roles not only in viral infectivity, but also in membrane fusion, and it is therefore a promising target for an anti-HIV antibody vaccine development (Montero et al, 2008; Zwick et al, 2005). MPER is weakly immunogenic since it's inaccessible to the immune system being masked in the unmodified HIV-1 envelope glycoprotein (Vassel et al, 2015). A number of strategies aimed at improving the accessibility of MPER and the consequent induction of neutralizing antibodies targeting the MPER bnAb epitopes have met with limited success. In this study, we used an RNA coliphage Qβ whose surface has been previously engineered by our research team to display all reported contiguous linear MPER epitopes corresponding to the bnAb 2F5, 4E10, Z13e1 and 10E8 through linking to the minor coat protein A1 of Qβ. This modification resulted to the surface display of 12 molecules of MPER per 25 nm hybrid phage particle thereby concentrating and improving the availability of the MPER based broadly neutralizing determinants for antibody recognition (Waffo et al, 2017). We noted that the recombinant QβMPERN phage reacted with their respective bnAbs in our ELISA antigenicity assay.

Our in-house ELISA assay was used to assess the antibody responses associated with HIV-I QBMPERN among HIV-I discordant couples from Southeast Nigeria. Most of the HIV-I infected individuals were in the age bracket of 31-40 years while the 51-60 years age bracket had the least infection of all age

groups. Most of the couples tested were also in the 31-40 years age bracket and women had more infection than men. Younger men had less infection than their female counterparts while older men were more infected than older women (51-60 years).. Our assay protocols were well optimized hence the strong response noted not only for the HIV-I infected but also for their seronegative partners. Dilutions for HIV-I QBMPERN were highly optimized to give good results. In-addition, dilutions for the serum samples, peroxidase labeled conjugate antibodies were equally optimized. Reaction temperatures and time were also optimized for best outcomes. Our ELISA assay offers an efficient and cost effective means for testing vaccine immunogens among large populations. It is cheaper than commercial ELISA kits and has more flexible use. Findings from this study shows that the hybrid phage the HIV-I QBMPERN could be used to develop commercial ELISA kits and test cassettes for detecting individuals who are exposed to HIV-I but remain uninfected.

CONCLUSION

This is the first report of an optimized ELISA assay for evaluating HIV-I QBMPERN associated broadly neutralizing antibody responses among HIV-I discordant couples in Nigeria. This study shows that the HIV-I QBMPERN could be harnessed for development of ELISA based reagents and test cassettes for screening HIV-I seronegative individuals who are highly exposed to HIV-I.

Conflict of interest: The authors declare that there is no conflict of interest.

Funding: This research was funded by **TETFund** Nigeria as part of an institution based research grant. We also appreciate the efforts of CIRCB, Yaoundé Cameroun in providing most of the reagents used for this study.

Limitation of study: We have used a small sample size in this study because of the unwillingness of some discordant couples to participate in the study

Author's contribution C.G.O., NGW, ECO, AGI, ABW, designed research, CGO, NGW, LNN, AAN, AL, HFO, DT, SHM, TFT, performed research, CGO, ECO, NGW, AAN, LNN, AL analysed data, C.G.O., N.G.W., E.C.O., COM, AGI, ANR, EIB, MPO, ASO wrote paper.

REFERENCES

Alain Bopda Waffo, Loveline Ngu, Rana L. Singleton, Timothy Egbo, Josué L. Simo, Carrie A. Sanders, Georgia E Ambada, Nadesh N. Nji, Apeh A. Ngoh, QianaL. Matthews, Boakai K. Robertson, Thibau F. Tchouangueu, Abel Lissom, Swapnil Bawage, ColinceTchadji, Arinze S. Okoli, Charles O. Esimone, Rebecca Chukwuanukwu, Wilfred Mbacham, Lazare Kaptue, Godwin W. Nchinda, (2017). Surface Engineering of Recombinant RNA Coliphage Qβ to Display gp41 MembraneProximal External-Region Epitopes from HIV-I. Journal of Clinical and Experimental Immunology; Volume 2 | Issue 1 | 1 of 15

Buchacher A, Predl R, Strutzenberger K, Steinfellner W, Trkola A, Purtscher M, et al. (1994) Generation of human monoclonal antibodies against HIV-1 proteins;

electrofusion and EpsteinBarr virus transformation for peripheral blood lymphocyte immortalization. AIDS research and human retroviruses 10: 359-369.

Hu H, He L, Hu Y, Jiang M, Yao K, Yang Y. (2008). Detection of Haemophilus influenzae type B DNA in a murine pneumonia model by in situ PCR. Journal of Medical Microbiology. ;57(Pt 10):1282–7.

Huang J, Ofek G, Laub L, Louder MK, Doria-Rose NA, Longo NS, et al. (2012) Broad and potent neutralization of HIV-1 by a gp41-specific human antibody. Nature 491: 406-412.

Kaplan SL, Mason EO Jr, Johnson G, Broughton RA, Hurley D, Parke JC Jr (1983).. Enzyme-linked immunosorbent assay for detection of capsular antibodies against Haemophilus influenzae type b: comparison with radioimmunoassay. J Clinical Microbiology.;18(5):1201–4.

Montero M, van Houten NE, Wang X, Scott JK (2008) The membrane-proximal external region of the human immunodeficiency virus type 1 envelope: dominant site of antibody neutralization and target for vaccine design. Microbiology and molecular biology reviews ☹MMBR) 72: 54-84, table of contents.

Morris L, Chen X, Alam M, Tomaras G, Zhang R, Marshall DJ, et al. (2011) Isolation of a human anti-HIV gp41 membrane proximal region neutralizing antibody by antigen-specific single B cell sorting. PloS one 6: e23532

Muster T, Steindl F, Purtscher M, Trkola A, Klima A, Himmler G, et al. (1993) A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. Journal of virology 67: 6642-6647.

Nelson JD, Brunel FM, Jensen R, Crooks ET, Cardoso RM, Wang M, et al. (2007) An affinity-enhanced neutralizing antibody against the membrane-proximal external region of human immunodeficiency virus type 1 gp41 recognizes an epitope between those of 2F5 and 4E10. Journal of virology 81: 4033- 4043.

Ofek G, Zirkle B, Yang Y, Zhu Z, McKee K, Zhang B, et al. (2014) Structural basis for HIV-1 neutralization by 2F5-like antibodies m66 and m66.6. Journal of virology 88: 2426-2441.

Riera L. (1985) Detection of Haemophilus influenzae type b antigenuria by Bactigen and Phadebact kits. Journal of Clinical Microbiology. ;21(4):638– 40. [PubMed: 3872880]

Singleton J, Osborn JL, Lillis L, Hawkins K, Guelig D, Price W, et al. (2014) Electricity-free amplification and detection for molecular point-of-care diagnosis of HIV-1. PloS one 9: e113693.

Skamel C, Aller SG, Bopda Waffo A (2014) In vitro evolution and affinity-maturation with Coliphage qbeta display. PloS one 9: e113069.

Torigoe H, Seki M, Yamashita Y, Sugaya A, Maeno M. (2007). Detection of Haemophilus influenzae by loop-mediated isothermal amplification (LAMP) of the outer membrane protein P6 gene. Jpn Journal of Infectious Diseases. ;60(1):55–8.

Vassell R, He Y, Vennakalanti P, Dey AK, Zhuang M, Wang W, et al. (2015)

Immunogens Modeling a Fusion-Intermediate Conformation of gp41 Elicit Antibodies to the Membrane Proximal External Region of the HIV Envelope Glycoprotein. PloS one 10: e0128562.

Zhu Z, Qin HR, Chen W, Zhao Q, Shen X, Schutte R, et al. (2011) Cross-reactive HIV-1-neutralizing human monoclonal antibodies identified from a patient with 2F5-like antibodies. Journal of virology 85: 11401-11408.

Zwick MB, Labrijn AF, Wang M, Spenlehauer C, Saphire EO, Binley JM, et al. (2001) Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. Journal of virology 75: 10892-10905.

Zwick MB, Jensen R, Church S, Wang M, Stiegler G, Kunert R, et al. (2005) Anti-human immunodeficiency virus type 1 (HIV-1) antibodies 2F5 and 4E10 require surprisingly few crucial residues in the membrane-proximal external region of glycoprotein gp41 to neutralize HIV-1. Journal of virology 79: 1252-1261.