Journal of Immunological Methods 466 (2019) 47–51

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Technical note

Development and validation of HIV-1 Multiplex Serology

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ARTICLE INFO

Keywords:
HIV
Serology
Seroepidemiology
Multiplex
Immunoassay
High-throughput

ABSTRACT

By inducing immunosuppression in infected patients, human immunodeficiency virus-1 (HIV-1) generates a favorable environment for opportunistic infections and the development of several human cancers. In order to detect individual serum or plasma HIV-1 antibody status for epidemiological studies, high-throughput HIV-1 Multiplex Serology was developed. Seven HIV-1 antigens were recombinantly expressed in *E. coli* as N-terminal glutathione-S-transferase (GST) fusion proteins that are bound to glutathione-coupled sets of beads with distinct fluorescent color. Combining all bead sets in a suspension array allowed for simultaneous detection of antibodies targeting structural, regulatory and accessory proteins expressed during HIV-1 infection. HIV-1 Multiplex Serology was validated with 244 reference sera whose HIV-1 serostatus had been pre-determined by screening microparticle immunoassay and confirmatory line immunoassay. The multifunctional protein GAG emerged as an excellent marker to determine HIV-1 serostatus with a specificity of 99% (95% CI 96%–100%) and sensitivity of 100% (95% CI 88%–100%). Seropositivity for multiple HIV-1 antigens appeared to be characteristic for HIV-1 infected individuals (median number of antigens recognized in reference assay positive sera: 4; median number of antigens recognized in reference assay negative sera: 0), indicating a broad immune response targeting also regulatory and accessory proteins which may be useful for the identification of antibody patterns specific for infection-associated disease stages. HIV-1 Multiplex Serology performs similarly to conventional HIV-1 serology but eliminates the need for a two-step screening approach with subsequent confirmation assay. Thus, this high-throughput method will facilitate large-scale epidemiological studies of the role of HIV-1 in cancer development.

1. Introduction

By inducing and maintaining immunosuppression, HIV-1 infection creates an environment that favors opportunistic infections that may result in tumor development. The development of cancer is associated with the progressive failure of the immune system and is among the major causes of morbidity and mortality in HIV-infected individuals (Newcomb-Fernandez, 2003).

The majority of cancer types exhibiting increased incidence rates among immunosuppressed individuals (e.g., anal cancer, non-Hodgkin lymphoma) is associated with viral infections (e.g., human papillomavirus, Epstein-Barr virus). Despite ubiquitous prevalence of several oncogenic viruses, tumor development is a very rare event in infected individuals, complicating the establishment of causal relationships. In order to investigate associations between HIV-1, opportunistic viral infections and tumorigenesis, large studies are needed to convey the statistical power needed to precisely describe the interplay of several pathogens in different populations and to facilitate the discovery of serological markers of disease development.

Conventional serologic assays are often based on initial screening techniques such as enzyme-linked immunosorbent assays (ELISA) with viral lysates, recombinant proteins and/or synthetic peptides, and confirmatory assays such as Western blot to eliminate false positives. These multi-step assay procedures naturally entail complex diagnostic algorithms and are mostly designed for the detection of a single agent. For large-scale studies of multiple infections where time, costs and sample volume are limited resources, high-throughput multiplexed serological analyses are mandatory.

Using fluorescent bead-based suspension array technology, we have developed Multiplex Serology to quantify antibodies to human papillomaviruses (Waterboer et al., 2005; Waterboer et al., 2006) and many other bacterial and viral infections e.g., (Michel et al., 2009;

Abbreviations: HIV-1, human immunodeficiency virus-1; GST, glutathione-S-transferase; MFI, Median Fluorescence Intensity; CI, confidence interval

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https://doi.org/10.1016/j.jim.2019.01.007
Received 27 September 2018; Received in revised form 20 December 2018; Accepted 16 January 2019
Available online 18 January 2019
0022-1759/

Dondog et al., 2015). This technique allows the quantification of specific serum antibodies against up to 100 different antigens simultaneously. The used antigens are recombinantly expressed glutathione-S-transferase (GST) fusion proteins with conserved conformational epitopes that are affinity-purified on glutathione-coupled polystyrene beads. Each antigen is bound to a different fluorescent bead set, and the bead sets are presented simultaneously to primary serum antibodies in a single reaction vessel. For each bead set, antibodies bound to the respective antigen are quantified separately. Here, we describe the development and validation of a multiplex assay for the detection of antibodies against seven structural, regulatory and accessory antigens of HIV-1 that can be easily incorporated into larger Multiplex Serology panels detecting antibodies against various pathogens simultaneously.

2. Methods

2.1. Human reference sera

For assay validation, archival sera with HIV-1 serostatus previously established at the routine diagnostic laboratory of the Saarland University Medical school (Germany) were re-analyzed by HIV-1 Multiplex Serology. HIV-1 reference serostatus was determined using the ARCHITECT HIV Ag/Ab Combo assay (Abbott), a chemiluminescent microparticle immunoassay (CMIA) designed for the simultaneous, qualitative detection of HIV-1 p24 antigen, antibodies against HIV-1 (group M and group O), and antibodies against HIV-2 in human serum and plasma. A positive result in the screening assay does not distinguish between the detection of HIV-1 p24 antigen, HIV-1 antibodies, or HIV-2 antibodies. Type-specific serum classification was performed using the confirmatory INNO-LIA HIV I/II Score assay (Innogenetics). This line immunoassay (LIA) determines the presence of antibodies against HIV-1 (including group O) and HIV-2 in human serum or plasma using recombinant proteins and synthetic peptides of HIV-1 (gp120, gp41, p31, p24, p17) and HIV-2 (gp105, gp36). For a positive HIV-1 test result, either two ENV bands (gp120 and gp41), one ENV band plus p24, or one ENV band plus any two other bands need to be present. False positive samples were defined as being positive in the screening assay but negative in the confirmatory assay.

2.2. Recombinant HIV-1 proteins

HIV-1 antigen-specific reference sequences were extracted from the HIV-1 BH10 genome sequence (M15654, GenBank), and a cloned genome plasmid served as template for PCR amplification of viral antigens. Primers were obtained from Eurofins MWG Operon (Ebersberg, Germany). Viral antigens were cloned into a modified pGEX4T3 vector yielding an open reading frame coding for an N-terminal GST domain followed by the individual insert and a C-terminal tag domain coding for the last eleven amino acids of the SV40 large T-antigen (Sehr et al., 2001).

Eight HIV-1 antigens were initially selected based on known or potential immunogenicity and expressed as double fusion proteins: GAG, ENV, TAT, REV, NEF, VIF, VPU and VPR (Table 1). GAG included all three domains of the GAG polyprotein precursor ($Pr55^{GAG}$). For ENV, both the first N-terminal 33 amino acids presenting the signal peptide, as well as gp41 starting from the cleavage site at amino acids 511–512 and its hydrophobic transmembrane domain were omitted to avoid low expression yields.

The efficiency of fusion protein expression was characterized by Western blotting. The use of GST- and tag-specific antibodies allowed the detection of proteins that contained both the N-terminal GST domain as well as the C-terminal tag epitope, revealing the extent to which full-length fusion protein had been expressed. Verification and concentration of full-length fusion proteins were determined by GST capture and anti-tag ELISA (Sehr et al., 2001). All HV-1 antigens were successfully expressed from sequence-confirmed plasmids except VPR.

Table 1
Characteristics of recombinant HIV-1 proteins expressed for HIV-1 Multiplex Serology. All genes are based on the genome sequence HIV-1 BH10 (gene accession number M15654). For spliced genes, the length of the transcript is given.

Antigen		Function	Genome region [nt]	Length [nt]	Protein region [aa]	NCBI Protein accession no.
GAG	Group-specific antigen	Cleavage into matrix, capsid and nucleocapsid.	112–1647	1536	1–512	AAA44201
ENV	Envelope glycoprotein	Surface subunit gp120. Fusion with host cell membrane.	5679-7112	1434	34–511	AAA44205
TAT	*Trans*-activating factor	Promotion of proliferation, differentiation, survival. Oncogenic.	5189-5403, 7734-7776	258	1–86	AAA44199
REV	Regulator of expression	Control of mRNA export rate.	5328–5403, 7734-8005	348	1–116	AAA44200
NEF	Negative factor	MHCI, MHCII and CD4 degradation, infectivity, cellular activation pathways.	8152-8520	369	1–123	AAA44206
VIF	Virus infectivity factor	Inhibition of APOBEC3G activity.	4399-4974	576	1–192	AAA44202
VPU	Viral protein U	Viral particle release, CD4 degradation.	5420-5662	243	1–81	AAA44204
VPR[a]	Viral protein R	Apoptosis, G_2 arrest.	4917-5150	234	1–78	AAA44203

MFI: Median Fluorescence Intensities.
[a] VPR could not be expressed.

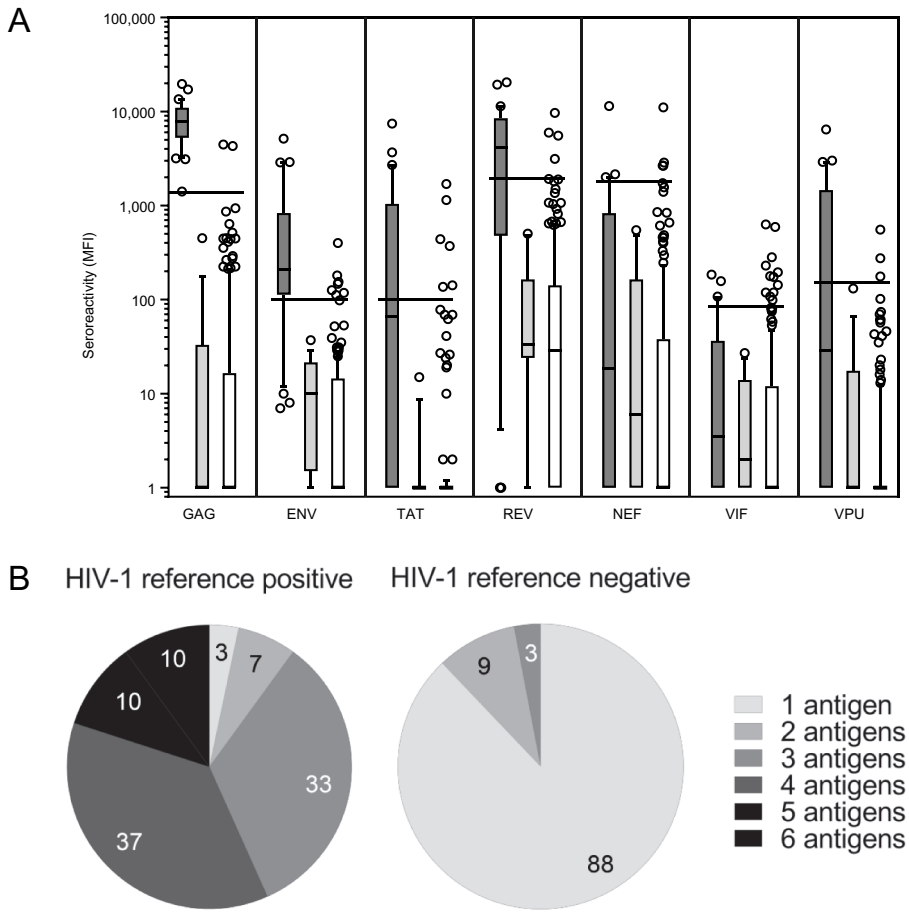

Fig. 1. Seroreactivity and seropositivity patterns determined with HIV-1 Multiplex Serology. A: Distribution of antibody reactivities measured in positive (n = 30, dark grey), false positive (n = 17, grey) and negative reference sera (n = 197, white) as classified by the reference assay. The boxes are delimited by the first and third quartile, the horizontal line inside the box represents the median. Whiskers show the 10th and 90th percentiles. Individual data points below and above the whiskers are drawn as circles. Horizontal lines across boxes indicate antigen-specific cut-offs. Differential seroreactivity between reference assay positive versus false positive, positive versus negative and negative versus false positive samples was evaluated for each antigen by one-way ANOVA and Tukey's post-test. For GAG, ENV, TAT, REV and VPU, seroreactivities in the positive reference group were significantly higher ($p < 0.0001$) than in false positives and negatives. For NEF, seroreactivities in the positive group were significantly higher than in the negative group ($p < 0.005$). All other statistical comparisons did not reveal statistically significant differences ($p \geq 0.05$). B: Proportions of sera recognizing one or more HIV-1 antigens in positive (n = 30) and negative reference sera reacting with at least one HIV-1 antigen (n = 33). In total, 181 reference assay negative and false positive sera (85%) did not react with any antigen. Numbers indicate percentages. MFI: Median Fluorescence Intensity.

Based on our observations and those by others (Bodéus et al., 1997), it is assumed that VPR interferes with bacterial cell growth, provoking the loss of the insert sequence as part of the bacterial survival strategy.

2.3. Multiplex Serology

Sera were analyzed for all seven successfully expressed HIV-1 antigens simultaneously by Multiplex Serology at serum dilution 1:100 as described previously (Waterboer et al., 2005; Waterboer et al., 2006). Briefly, HIV-1 GST-tag double fusion proteins were affinity-purified from crude lysates through binding to glutathione-casein-coated fluorescence-labeled polystyrene beads. Loading of each antigen onto its own distinct bead set and subsequent mixing of bead sets created a suspension array in which all different antigens were simultaneously presented to the primary antibodies of the serum to be tested. A Luminex 200 flow cytometer (Luminex Corp.) was used to identify the bead color, i.e. the corresponding antigen against which serum antibodies are detected, and to quantify the fluorescent reporter conjugate streptavidin-R-phycoerythrin bound to biotinylated anti-human IgM/IgG/IgA secondary antibodies (1:1000, #109-065-064, Jackson Immunoresearch) as median fluorescence intensity (MFI) of at least 100 beads per set per serum.

Antigen loading efficiency was monitored via detection of the C-terminal tag with biotinylated mouse anti-tag antibody (Dondog et al., 2015), to reflect the extent of antigen loading on each bead set (Waterboer et al., 2006). For the correction of background arising from autofluorescence of the beads and unspecific binding of the secondary antibody to the antigens, one well per plate did not contain serum (negative control). One bead set was loaded with GST-tag without viral protein to determine and subtract sample-specific background.

2.4. Data processing and statistical analyses

Net MFI values were calculated by subtracting unspecific background (negative controls) and reactivity towards GST-tag (sample-specific background) from raw MFI values. Antigen-specific cut-off values were derived by grouping antibody reactivities according to reference assay serostatus and maximizing sensitivity and specificity analogous to Receiver Operating Characteristics analysis, with a predefined minimum specificity of 90%. Reference assay false positives and negatives were jointly classified as reference assay negatives for statistical evaluation. Seropositivity for a given antigen in HIV-1 Multiplex Serology was defined as antibody reactivity above the antigen-specific cut-off. Cohen's *kappa* (k) statistics as a metric for agreement as well as sensitivity and specificity with their corresponding 95% confidence intervals (CI) were calculated to estimate assay performance. Pearson's r was calculated as a metric for antibody correlation. One-way ANOVA and Tukey's post-test were used to evaluate differences in seroreactivity (net MFI values) with individual antigens between HIV-1 positive and false positive, positive and negative or negative and false positive samples. *P*-values below 0.05 were considered statistically significant. Figures and statistical analyses were generated with GraphPad Prism 7.

3. Results and discussion

3.1. HIV-1 Multiplex Serology validation and concordance with the reference assay

For validation of HIV-1 Multiplex Serology, a panel of 244 reference sera classified by CMIA and LIA was used. Of these sera, 30 had been

Table 2
Antigen-specific seropositivity in HIV-1 Multiplex Serology and statistical performance. Antigen-specific serostatus determined by HIV-1 Multiplex Serology was compared with serostatus of the reference assay which classified sera positive (n = 30), false positive (n = 17), or negative (n = 197). Agreement (κ), specificity and sensitivity are depicted. Reference assay false positives and negatives were jointly classified and analyzed as reference assay negatives. Antigen-specific cut-offs were derived from pre-classified serostatus maximizing specificity and sensitivity with a pre-defined minimum specificity of 90%.

	Cut-off [MFI]	HIV-1 positive (N = 30) n (%)	HIV-1 false positive (N = 17) n (%)	HIV-1 negative (N = 197) n (%)	κ (95% CI)	Specificity (95% CI)	Sensitivity (95% CI)
GAG	1400	30 (100)	(0)	2 (1)	0.96 (0.91–1.00)	99 (96–100)	100 (88–100)
ENV	100	25 (83)	(0)	7 (4)	0.77 (0.66–0.90)	97 (93–99)	83 (65–94)
TAT	100	15 (50)	(0)	6 (3)	0.54 (0.37–0.71)	97 (93–99)	50 (31–69)
REV	1930	19 (63)	(0)	4 (2)	0.68 (0.53–0.83)	98 (95–99)	63 (44–80)
NEF	1800	6 (20)	(0)	4 (2)	0.25 (0.07–0.44)	98 (95–99)	20 (8–39)
VIF	85	4 (13)	(0)	12 (6)	0.10 (0.06–0.25)	94 (90–97)	13 (4–31)
VPU	150	13 (43)	(0)	3 (2)	0.53 (0.35–0.70)	99 (96–100)	43 (26–63)

N: number of sera in the reference assay as classified.
CI: confidence interval.

found positive (CMIA and LIA positive), 17 false positive (CMIA positive but LIA negative) and 197 negative (CMIA negative).

Grouping of antigen-specific antibody reactivities obtained from HIV-1 Multiplex Serology according to reference assay status revealed clear separation between positives versus false positives or positives versus negatives for GAG, ENV, TAT, REV and VPU, and differences in reactivities (MFI) were highly significant ($p < 0.0001$) (Fig. 1A). In contrast to GAG and ENV where MFI values of the positive group rarely overlapped with those of the false positive or negative group, the difference between reference groups for TAT, REV and VPU was much less distinct. Despite extensive overlap between the positive and the negative group for NEF, this difference was significant ($p < 0.005$); however, there was no significant difference between the positive and the false positive group. Antibody reactivities against VIF were not significantly different between the three groups of reference sera. Significant differences between false positives and negatives were not observed for any antigen.

Of the 30 HIV-1 reference assay positive sera, all (100%) were seropositive for GAG, and 25 (83%) for ENV (Table 2). The reference assay positive sera also reacted with regulatory and accessory proteins, ranging from 4 (13%) with VIF to 19 (63%) with REV. All but one of the sera reacting with GAG reacted with at least one other antigen. Similarly, all sera seropositive to ENV were seropositive to GAG plus at least one other antigen.

Among negative reference sera, seropositivity to any of the antigens was rare, ranging from 6% for VIF to 1% for GAG (Table 2). In total, 164 reference assay negative sera (83%) did not react with any antigen. In contrast to positive reference sera, the seven negative reference sera testing positive for ENV antibodies were single positive, i.e. seronegative to all other antigens. Interestingly, none of the false positive reference sera was recognized by any antigen in HIV-1 Multiplex Serology. These seropositivity patterns resulted in differential levels of agreement with the reference assay ranging from no significant difference from chance for VIF to almost perfect agreement for GAG (k: 0.10–0.96; Table 2).

Antibody reactivities for GAG in positive reference sera yielded MFI values between 1414 and 19609 (median: 7768 MFI), whereas all but two negative (n = 195) and all false positive sera (n = 17) were found below 950 MFI. The fact that the range of MFI values was very similar for false positive and negative reference sera implicates a higher specificity of HIV-1 Multiplex Serology GAG compared to the reference assay while maintaining the same sensitivity (Fig. 1A, Table 2). ENV reactivities were spread across a large range among reference assay positive sera (8 to 5154 MFI; median: 209 MFI). Despite the reported high immunogenicity of ENV (Gils and Sanders, 2013), five out of 30 positive reference sera (17%, cut-off: 100 MFI) were not recognized, yielding a sensitivity and specificity of ENV alone of 83% and 97%, respectively. In contrast, the confirmatory LIA found ENV antibodies (both anti-gp120 and anti-gp41) in all sera tested HIV-1 positive. This divergence may be attributable to a potentially decreased antigenicity of the bacterially expressed recombinant fusion protein due to lacking glycosylation, and/or the absence of gp41 in Multiplex Serology.

Seropositivity against GAG alone (cut-off: 1400 MFI) was defined as criterion for HIV-1 seropositivity and conveyed 100% sensitivity and 99% specificity. Only two of the 197 negative reference sera were discordantly classified as HIV-1 positive by GAG. These two sera reacted only with GAG and no other antigen. Of the reference positive sera, one serum also demonstrated seropositivity against GAG only. Thus, it may be speculated that these two discordant sera are in fact truly HIV-1 positive and were missed by the reference assay. Alternatively, specificity could be further enhanced by redefining the diagnostic algorithm: Instead of GAG alone, GAG and any other antigen may be used to define HIV-1 seropositivity, yielding 100% specificity. This alternate algorithm, however, misses one positive serum in the positive reference group, diminishing sensitivity to 96%. When repeating the analysis at serum dilution 1:1000, a GAG AND ENV combination (cut-off$_{GAG}$: 1722, cut-off$_{ENV}$: 100) maintained high specificity of 100% (95% CI 98–100%) and sensitivity of 90% (95% CI 74–98%) (data not shown).

3.2. Antibody patterns in HIV-1 Multiplex Serology

Multiple seropositivity against HIV-1 antigens was a ubiquitous feature among positive reference sera, as 29 (97%) reacted against at least two antigens (Fig. 1B), of which 27 (90%) were found to be positive for antibodies against at least three different HIV-1 antigens. Among negative and false positive reference sera, the majority of sera reacting to at least one antigen (n = 33, 15%) was single positive (n = 29, 88%). Thus, multiple seropositivity appears to be a common antibody pattern among HIV-1 infected patients, indicating a broad immune response targeting also regulatory and accessory proteins.

While antibody reactivities against ENV in positive reference sera coincided with GAG positivity, the prevalence of antibodies targeting HIV-1 antigens other than GAG and ENV was heterogenous (Table 2), and correlation with GAG ranged from $r = -0.04$ for TAT to $r = 0.77$ for REV (Supplementary Fig. 1). Although reactivities towards these antigens clearly accumulated in positive reference sera, reactivities were also observed in 1–6% of negative sera. Despite high specificities ranging from 94% (VIF) to 99% (VPU), the additional antigens lacked the necessary sensitivity in order to serve as diagnostic markers for HIV-1 infection, with sensitivities ranging from 13% (VIF) to 63% (REV) (Table 2).

4. Conclusion

Multiplex Serology is a high-throughput technology for the determination of serum antibody prevalence based on the presentation of bead-coupled recombinant antigens. It is unique in its ability to

quantify specific antibodies directed against conformational epitopes of a large set of proteins simultaneously. This technology was successfully validated for seven HIV-1 antigens. GAG emerged as an excellent marker for the determination of HIV-1 status, and multiple seropositivity appears to be a hallmark of HIV-1-infected patients, indicating the establishment of a broad immune response targeting also regulatory and accessory proteins. It remains to be determined whether antigenic patterns besides GAG and ENV help further characterize the stage of infection and add to disease prediction. Easy implementation into broad Multiplex Serology panels covering other viral or bacterial infections will greatly facilitate and accelerate investigation of the mechanism and circumstances in which HIV-1 infection may contribute to the development of certain types of cancer - whether through severely impaired control of persistent tumorigenic viruses or opportunistic infections. HIV-1 Multiplex Serology cannot replace the conventional diagnostics approach as it does not detect infection with HIV-2. In addition, the window period between infection and a positive test result is longer in comparison to Nucleic Acid Tests (NAT). However, the combination of high specificity and sensitivity in a single assay eliminates the need for confirmatory assays, rendering HIV-1 Multiplex Serology superior to conventional two-assay diagnostics for large-scale epidemiological studies of HIV-1.

Disclosure of potential conflicts of interest

No potential conflicts of interest are disclosed.

Grant support

None.

Acknowledgments

The authors would like to thank Ute Koch und Monika Oppenländer for excellent technical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.01.007.

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