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Multi-laboratory assessment of EBV serologic assays: the case for standardization

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Abstract

Background

IgA antibodies targeting Epstein-Barr virus (EBV) have been proposed for screening for nasopharyngeal carcinoma (NPC). However, methods vary, and antigens used in these assays differ considerably between laboratories.

Methods

To enable formal comparisons across a range of established EBV serology assays, we created a panel of 66 pooled serum and 66 pooled plasma samples generated from individuals with a broad range of IgA antibody levels. Aliquots from these panels were distributed to six laboratories and tested by 26 assays measuring antibodies against VCA, EBNA1, EA-EBNA1, Zta, or EAd antigens. We estimated the correlation between assay-pairs using Spearman coefficients (continuous measures) and percentage agreement (positive versus negative using pre-defined positivity cutoffs by each assay developer/manufacturer).

Results

While strong correlations were observed between some assays, considerable differences were also noted, even for assays that targeted the same protein. For VCA-IgA assays in serum, two distinct clusters were identified, with the median Spearman coefficient of 0.41 (range: 0.20 – 0.66) across these two clusters. EBNA1-IgA assays in serum grouped into a single cluster with the median Spearman coefficient of 0.79 (range: 0.71 – 0.89). Percentage agreements varied broadly for both VCA-IgA (12% – 98%) and EBNA1-IgA (29% – 95%) assays in serum. Moderate-to-strong correlations were observed across assays in serum that targeted other proteins (correlations range: 0.44 – 0.76). Similar results were noted for plasma.

Conclusion

Standardization of EBV serology assays is needed to allow for comparability of results obtained in different translational research studies across laboratories and populations.

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Introduction

Assays that measure antibody responses to Epstein-Barr virus (EBV) have become increasingly important tools for studying and diagnosing nasopharyngeal carcinoma (NPC) and other research (1, 2). Several studies have shown that individuals with elevated levels of antibody responses against EBV antigens (particularly IgA responses) are at increased risk for the development of NPC (3-15). In NPC endemic areas such as Southern China, EBV IgA antibody testing has been proposed for general population screening to triage individuals to further clinical evaluation aiming at the early detection and treatment of NPC (4, 7, 16, 17). However, recent studies have elucidated the underlying (epitope) complexity of anti-EBV antibody responses, and this needs to be considered in order to achieve standardization amongst the community (2).

IgA antibodies against EBV capsid antigen (VCA-IgA) and EBV nuclear antigen 1 (EBNA1-IgA) are the two EBV serological markers most frequently considered for screening purposes (4, 7, 16-18). However, several assays that measure VCA-IgA and EBNA1-IgA exist, and efforts to standardize these EBV assays have been limited, making it difficult to compare results across studies that utilize different assays. To date, no studies have directly compared VCA- or EBNA1-IgA results from the various assays used in different laboratories globally to define interassay agreement or to assess whether the same humoral immune response is being measured by each assay. As such markers have been proposed for use in NPC early-detection screening programs. Understanding the relationship between existing commercial and research assays is needed to interpret the published literature. Evaluation of the correlation and percentage agreement between assays represents an important initial step toward the standardization for assays intended for clinical use.

To measure agreement between assays measuring antibodies against EBV, we conducted a study in which pools of serum and plasma from individuals with a range of expected antibody levels were created and blindly distributed to six different laboratories for testing. We initially focused on assays that measure antibodies against VCA and EBNA1 because those are the two main EBV antigens targeted for antibody tests considered for EBV screening purposes. Herein, we described the various laboratories' methods and correlation/agreement between assays. For completeness, we also included assays that measure antibodies against other EBV proteins (*e.g.*, early D antigen [EAd] and Zta) to understand the correlations between assays that measure antibodies against these different proteins.

Methods

<u>*Source population*</u>

This panel of EBV serology standards was created by capitalizing on biospecimen resources from ongoing and completed studies conducted in Taiwan (10, 19) between 1991 and 2016. Serum and plasma samples were prepared within 24 hr of collection and stored frozen at -80°C until analysis. These studies were reviewed/approved by the National Cancer Institute Special Studies Institutional Review Board and the National Taiwan University Institutional Review Board. Written informed consent was obtained for all participants.

<u>Creating pools for testing</u>

To create a resource with sufficient volume to permit testing by multiple assays in multiple laboratories, pooling samples across individuals was required. We created both serum and plasma pools with different individuals contributing samples for serum pools and plasma pools because of limited specimen availability from the previous studies. To ensure that a broad distribution of IgA antibody responses was retained after pooling, blood samples from individuals with similar expected IgA responses were pooled whenever possible. IgA antibody titers at collection were retrieved from participants' medical files or experimental records at collection, based on different IgA assays in routine clinical use at the time each of the studies was conducted. Briefly, a total of 66 pooled serum samples and 66 pooled plasma samples were generated from an average of two individuals (range: 1-5), of which 22 pooled serum/plasma samples were created from 1) NPC cases (representing samples with potentially elevated

IgA antibody titers) and non-NPC cases with known high levels of IgA antibodies against EBV, 2) general population controls from a previously conducted NPC case-control study (representing samples expected to have low IgA antibody titers) and hospital outpatients with known low levels of IgA antibodies against EBV, and 3) unaffected individuals from an ongoing NPC multiplex family study (representing individuals at high risk of developing NPC).

Plate batching of pools

Participating laboratories were provided with one aliquot (range: 25µl – 150µl) of each sample without knowledge of whether the sample came from high-risk or low-risk pools. We also included approximately 20% randomly selected, blinded duplicate samples (N=14) to assess within-assay intraclass correlation coefficients (ICCs) and coefficients of variation (CV). All samples were randomly distributed on the plate and sent to participating laboratories in individual cryovials.

Assays performed

Six independent laboratories agreed to test serum and/or plasma specimens using research or commercial assays (enzyme-linked immunosorbent assay [ELISA] or Luminex assays). Of the 26 assays, two VCA-IgA assays (A2.1 and A2.2) and two EBNA1-IgA assays (A9.1 and A9.2) comprised commercial assays purchased from the same company but tested in different laboratories with different pre-defined positivity cutoffs. No special instructions were given to the laboratories regarding the handling or testing of these specimens. Details of each assay, including information on sample dilution, antigens targeted, amino acid sequences, and whether the assays were designed to capture IgA, IgG, or IgG/IgA/IgM are provided in **Supplementary Materials** and **Supplementary Table 1**. In total, we included eight assays designed to measure antibodies against VCA, of which six assays were designed to detect IgA, one assay was designed to detect IgG/IgA/IgM, and one assay was designed to detect IgG. Nine assays designed to measure antibodies against EBNA1, of which six assays were designed to detect IgA, two assays were designed to detect IgG/IgA/IgM, and one assay was designed to detect IgG. Nine assays were designed to measure antibodies against other antigens (*i.e.*, EA-EBNA1, Zta and EAd), of which two assays were designed to detect IgA against EA-EBNA1 combined, four assays were designed to detect antibodies against EAd (two for IgA, one for IgG/IgA/IgM and one for IgG), and three assays were designed to detect antibodies against Zta (two for IgA and one for IgG/IgA/IgM).

Statistical Analysis

We first utilized the blinded duplicate pools included in our panel to estimate reproducibility of the 26 assays performed as part of our effort. For each specimen type (*i.e.*, serum or plasma), assays were clustered according to their Spearman correlations using unsupervised hierarchical clustering with Euclidean distance and complete linkage (20). Correlation coefficients of larger than 0.7, between 0.5 and 0.7, and less than 0.5 were considered to be strong, modest, and weak correlations, respectively (21). We also estimated percentage agreement and Kappa value between assay pairs using pre-defined positivity cutoffs for IgA assays as these IgA assays have been proposed for screening for NPC (**Supplementary Table 1**).

Analyses were performed using R Statistical Software (Foundation for Statistical Computing, Vienna, Austria). All statistical tests were 2-sided, and $P < 0.05$ was considered statistically significant.

Results

After quality control, we excluded from further consideration six assays evaluating serum (*i.e.*, Assays A18, A19, A21, A22, A23, and A24) and five evaluating plasma (*i.e.*, Assays A3, A9.2, A14, A23, and A24) with ICC<0.8 or CV>20% (**Table 1**). Among assays measuring antibodies against VCA, we included eight assays (six IgA, one IgG, and one IgG/IgA/IgM) for serum and seven assays (five IgA, one IgG, and one IgG/IgA/IgM) for plasma. Among assays measuring antibodies against EBNA1, we included nine assays (six IgA, one IgG, and two IgG/IgA/IgM) for serum and seven assays (five IgA, one IgG, and one IgG/IgA/IgM) for plasma. Among assays measuring antibodies against other antigens (*i.e.*, EAd and Zta), we included two assays (all IgA) for serum and six assays (three IgA, one IgG, and two

IgG/IgA/IgM) for plasma in the analysis. The average response levels are summarized in **Table 1** and results stratified by our three pre-defined groups are shown in **Supplementary Table 2**.

Antibodies against VCA

The correlations between assays measuring antibodies against VCA in serum are presented in **Figure 1A**. A total of three clusters were identified. Correlations tended to be higher within rather than across immunoglobin classes (Clusters #1 and #2 vs. Cluster #3; Cluster #3 representing IgG and IgG/IgA/IgM). IgA only assays grouped into two clusters: Cluster #1 included three research assays measuring the same antigen (VCA-p18; [Assays A1, A4, and A5] sequences illustrated in **Supplementary Figure 1**) with a median Spearman coefficient of 0.85 (range: 0.85 – 0.87); Cluster #2 included two commercial assays (assays A2.1/A2.2 and A3. Assays A2.1 and A2.2 were purchased from the same company but tested by two different labs) with a median Spearman coefficient of 0.71 (range: 0.64 – 0.97). Weak-to-moderate correlations were observed among IgA assays across Clusters #1 and #2, with a median Spearman coefficient of 0.41 (range: 0.20 – 0.66). The lowest correlation was observed between assays A2.2 and A5 (Spearman coefficient = 0.20).

Among IgA only assays, the percentage agreement for serum varied considerably from 12% - 98% (Kappa values ranged from -0.03 to 0.9, **Table 2)**. Higher agreements were observed between assays that clustered together in **Figure 1** (e.g., between assays A2.1 and A2.2, 95%; and between assays A4 and A5, 98%). By contrast, lower agreements were observed between assays that clustered separately (**Figure 1**, e.g., between assays A1 and A3, 12%; and between assays A3 and A5, 15%).

Antibodies against EBNA1

The correlations between assays measuring antibodies against EBNA1 in serum are presented in **Figure 1B**. Again, among three clusters that were identified, correlations tended to be higher within rather than across immunoglobin classes (Cluster #1 vs Clusters #2 and #3; Clusters #2 and #3 representing IgG/IgA/IgM and IgG). In contrast to observations made for VCA, all IgA only assays grouped into a single cluster (sequences illustrated in **Supplementary Figure 2**), with a median Spearman coefficient of 0.79 (range: 0.71 – 0.89). However, a wide range of percentage agreement (29% – 95%, Kappa values ranged from 0.1 to 0.9, **Table 3)** was observed for these IgA assays.

Antibodies against other EBV antigens (i.e., Zta and EAd)

To understand the correlations between assays measuring antibodies against distinct EBV proteins (i.e., Zta, EAd, VCA and EBNA1,) we compared results from assays targeting Zta and EAd (sequences illustrated in **Supplementary Figure 3**) against representative assays targeting VCA and EBNA1. Specifically, for this evaluation we included one IgA assay for each of the two clusters identified for VCA IgA (assays A1 and A2.1) and one assay from the single cluster identified for EBNA1 IgA (assay A8). The correlations between those assays in serum are shown in **Figure 1C**. Weak-to-moderate correlations were observed for IgA assays, with a median Spearman coefficient of 0.60 (range: 0.44 – 0.76).

Results in plasma

Similar correlations were observed in serum as in plasma when comparisons were made across assays and results are presented in **Supplementary Figure 4 and Supplementary Tables 3-4**.

Discussion

IgA antibodies against EBV VCA and EBNA1 have been proposed to facilitate diagnosis and early detection of NPC in high incidence regions (9, 17, 18). However, there has been very little effort to standardize the assays being considered for such programs and to understand the similarities and differences in their performance. Herein, we report the first study to directly compare assays designed to measure these antibodies. Although we observed high correlation and agreement between some assays, our results demonstrate wide variability among the assays evaluated when assays were compared with respect to both antibody levels and serostatus. Such variability could be caused by differences in targeted antigens, detection methods, and dynamic range of assays. These findings highlight the need for more formal attempts to validate and standardize EBV serology assays that are being considered or used for population screening or clinical diagnosis aimed at the early detection of NPC.

In the present study, clear differences were observed for assays designed to detect antibodies against VCA. Although a low agreement between assays designed to measure different Ig classes (IgG vs. IgA) was expected (22), two distinct clusters of IgA assays were noted. For these two clusters, good agreement was noted for assays contained within a cluster while poor agreement was observed for assays contained across clusters. The high correlation within clusters is likely explained by sharing of antigens/epitopes targeted by these assays (e.g., assays A1, A4, and A5 targeted VCA-p18, one of six proteins comprising the EBV viral capsid), although in some instances (assays A2.1, A2.2, and A3) we could not confirm this fact since information on target probes was not disclosed by the assay developer/manufacturer. The EBV VCA is a complex containing major capsid protein (p160; BcLF1), small capsid protein (VCA-p18, BFRF3), scaffold protein (VCA-p40, BdRF1), tegument protein p23 (BLRF2), glycoproteins gp125/110 (BALF4), and gp350/220 (BLLF1) (2). The immunodominant and virus-specific antigenic domain of VCA-p18 has been mapped and is located in its C-terminus (AA 110-176), whereas such domain is less clear for other VCA complex proteins (2). It is expected that different VCA components will contain distinct immunodominant domains, induce different levels of antibody response, and have different diagnostic performance. Moving forward, reporting of probe sequences used to measure EBV VCA antibodies will be important to facilitate interpretation of results across studies.

For EBNA1, we noted poor agreement for assays designed to detect different Ig classes but better agreement for assays designed to detect IgA, suggesting that these assays target similar epitopes. In fact, review of the probe sequences used to capture antibodies against EBNA1 revealed overlap across all assays for AA 382-404. This is consistent with reports that an immunodominant epitope of the EBNA1 protein (BKRF1, the major antigenic component of the EBNA complex), is located within AA 390-450 (2, 13, 23). Nonetheless, it is important to note that despite the high correlation observed for EBNA1 IgA assays, the range of percentage positive agreements between these assays was wide, suggesting varying sensitivities or thresholds for defining a positive response. The seropositivity cut-point we applied for each assay was predefined by the assay developers/manufacturers. These different assay positivity rates further highlight the need for careful validation and standardization of these assays in the future.

The moderate correlations for assays measuring IgA antibodies against different EBV proteins (VCA, EBNA1, EAd and Zta) was included in this report for completeness and provides a useful benchmark when evaluating levels of agreement for VCA and EBNA1 assays. Rates of agreement across protein targets were consistent with previous findings (22). The elevated levels of anti-EBV antibodies could indicate the ongoing viral lytic activity (reactivation) and a potential lack of control over the virus in general. Noteworthy is the fact that levels of agreement observed across proteins (expected to be modest) overlap with those noted within proteins (expected to be high for well standardized and characterized assays), again highlighting the need for further assay standardization in the future.

Strengths of our study included carefully selected pools meant to represent the entire expected range in antibody levels, direct comparison of assays using these pools, inclusion of many assays and laboratories. However, our results should be interpreted in light of some limitations. First, serum and plasma samples were not collected from the same individuals, which precludes us from formally comparing the antibody level and its correlation between serum and plasma based on paired samples. Second, information on the nature of EBV antigen used was missing for a few assays, which precludes us from further exploring the factors causing variability across different assays.

In conclusion, using a carefully-defined panel of serum and plasma samples distributed among multiple reference laboratories, we report high agreement for some assays designed to measure antibodies against same EBV antigens. However, we also observed considerable variability in the agreement between assays designed to measure antibodies against EBV VCA and EBNA1, both with respect to their correlation and to their reported positivity rates. Our study highlights the need for more systematic standardization of these assays and for the development of an international standard for measuring these antibody responses in serum or plasma. Such efforts are pre-requisites for the formal evaluation and quantitation of the performance of these assays in clinical practice or for population-based screening aimed at the early detection of NPC in high incidence regions.

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Figure Legends

Figure 1. Unsupervised hierarchical clustering based on Spearman correlation coefficient between assays measuring anti-EBV antibodies in serum. **A)** Antibodies against EBV capsid antigen (VCA); **B)** Antibodies against EBV nuclear antigen 1 (EBNA1); **C)** Antibodies against Zta (ZEBRA), early D antigen (EAd), VCA, and EBNA1. Red depicts a strong positive correlation, and blue indicates a weak correlation.

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Table 1. Summary of titer for assays testing anti-EBV antibodies.

Assay	Antigen	Antibody type	Method	Unit	Serum		Plasma	
					Median (IQR) [a]	Min-Max	Median (IQR) [a]	Min-Max
VCA								
A1	VCA-p18	IgA	ELISA	OD	4.28 (5.61)	1.25-22.58	2.41 (3.63)	0.53-17.12
A2.1	VCA	IgA	ELISA	relative OD	1.18 (2.92)	0.19-14.71	0.7 (2.15)	0.15-13.5
A2.2	VCA	IgA	ELISA	OD	0.92 (1.98)	0.19-10.8	0.66 (2.06)	0.2-11
A3 [b]	VCA	IgA	ELISA	OD	0.07 (0.16)	0.01-0.9	N/A	N/A
A4	VCA-p18	IgA	Luminex	MFI	1737 (2726.75)	15-10615	1105 (2517.25)	6-10231
A5	VCA-p18	IgA	Luminex	MFI	1682.5 (2932.5)	102-16524	1082 (2317)	45-12190
A6	VCA-p18	IgG	Luminex	MFI	11466.25 (3455.62)	1256.5-21006	13130.25 (6511.5)	1661-19609
A7	VCA-p18	IgG/IgA/IgM	Luminex	MFI	3065 (2764)	377-13253	2444 (2073.5)	164-7492
EBNA1								
A8	EBNA1	IgA	ELISA	OD	1.12 (6.6)	0.7-25.5	0.87 (4.04)	0.44-17.65
A9.1	EBNA1	IgA	ELISA	relative OD	0.44 (2.58)	0-5.07	0.25 (2.01)	0.00-5.04
A9.2 [b]	EBNA1	IgA	ELISA	OD	0.13 (1.09)	0-2.65	N/A	N/A
A10	EBNA1	IgA	Luminex	MFI	58 (1335.5)	5-1987	38 (1353.75)	35796
A11	EBNA1	IgA	Luminex	MFI	277 (1434.75)	52-4994	185.5 (1137.5)	19725
A12	EBNA1	IgA	Luminex	MFI	119 (966.88)	26-4131.5	71.5 (658.25)	30-3403
A13	EBNA1	IgG	Luminex	MFI	10569.75 (4737.62)	345-16185	10938 (7585.25)	290-17357.5

A14 [b]	EBNA1	IgG/IgA/IgM	Luminex	MFI	3109 (2162)	89-17673	N/A	N/A
A15	EBNA1	IgG/IgA/IgM	Luminex	MFI	7741.5 (3384.75)	836-17545	5816.5 (6303)	257-17412
Other antigens								
A16	EAd	IgA	Luminex	MFI	147 (2003.75)	1-13243	48.5 (2046.25)	1-13982
A17	EAd	IgA	Luminex	MFI	96 (75.25)	37-2654	62.75 (85.25)	29-14150
A18 [b]	EAd	IgG	Luminex	MFI	N/A	N/A	90.75 (610)	29-7846
A19 [b]	EAd	IgG/IgA/IgM	Luminex	MFI	N/A	N/A	309.5 (1518)	1-15095
A20	Zta (ZEBRA)	IgA	Luminex	MFI	30.5 (237)	1-5024	18 (537.25)	1-8591
A21 [b]	Zta (ZEBRA)	IgA	ELISA	OD	N/A	N/A	0.08 (0.11)	0.02-1.73
A22 [b]	Zta (ZEBRA)	IgG/IgA/IgM	Luminex	MFI	N/A	N/A	268.5 (892)	1-6309
A23 [b]	EA-EBNA1	IgA	ELISA	OD	N/A	N/A	N/A	N/A
A24 [b]	EA-EBNA1	IgA	ELISA	OD	N/A	N/A	N/A	N/A

Abbreviations: EBV (Epstein-Barr virus); EAd: early D antigen; EBNA1: EBV nuclear antigen 1; ELISA, enzyme-linked immunosorbent assay; MFI, median fluorescence intensity; OD, optical density; VCA: EBV capsid antigen.

a. Median level is based on 66 pooled samples.

b. Results are presented as "N/A" for assays with intraclass correlation coefficient (ICC) <0.8 or coefficients of variation (CV) >20%.

344

Table 2. Percentage agreement (Kappa) for assays detecting IgA antibodies against VCA in serum. [a]

Assay	A1	A2.1	A2.2	A3	A4	A5
A1 [b]	100	52 (N/A)	47 (N/A)	12 (N/A)	95 (N/A)	97 (N/A)
A2.1	--	100	95 (0.9)	61 (0.2)	50 (-0.03)	52 (0.002)
A2.2	--	--	100	65 (0.3)	48 (0.02)	50 (0.05)
A3	--	--	--	100	17 (0.01)	15 (0.009)
A4	--	--	--	--	100	98 (0.8)
A5	--	--	--	--	--	100

Abbreviations: VCA: Epstein-Barr virus capsid antigen.

a. Cells with duplicated information are presented as "--".

b. All samples were defined as positive by Assay A1. No Kappa value can be estimated, and results are presented as "N/A".

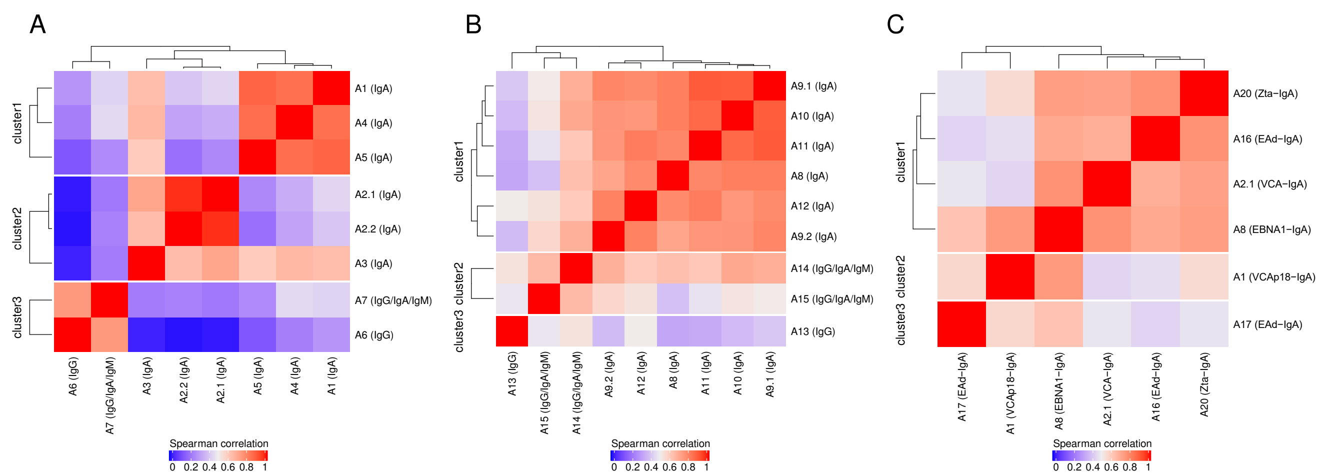
Table 3. Percentage agreement (Kappa) for assays measuring IgA antibodies against EBNA1 in serum. [a]

Assay	A8	A9.1	A9.2	A10	A11	A12
A8	100	67 (0.7)	76 (0.9)	73 (0.8)	62 (0.2)	68 (0.7)
A9.1	--	100	88 (0.8)	94 (0.8)	29 (0.1)	92 (0.9)
A9.2	--	--	100	94 (0.9)	41 (0.2)	89 (0.7)
A10	--	--	--	100	35 (0.2)	95 (0.7)
A11	--	--	--	--	100	30 (0.1)
A12	--	--	--	--	--	100

Abbreviations: EBNA1: Epstein-Barr virus nuclear antigen 1.

a. Cells with duplicated information are presented as "--".

Figure 1


A
cluster1
cluster2
cluster3
A1 (IgA)
A4 (IgA)
A5 (IgA)
A2.1 (IgA)
A2.2 (IgA)
A3 (IgA)
A7 (IgG/IgA/IgM)
A6 (IgG)
Spearman correlation
0 0.2 0.4 0.6 0.8 1
B
cluster1
cluster2
cluster3
A9.1 (IgA)
A10 (IgA)
A11 (IgA)
A8 (IgA)
A12 (IgA)
A9.2 (IgA)
A14 (IgG/IgA/IgM)
A15 (IgG/IgA/IgM)
A13 (IgG)
Spearman correlation
0 0.2 0.4 0.6 0.8 1
C
cluster1
cluster2
cluster3
A20 (Zta−IgA)
A16 (EAd−IgA)
A2.1 (VCA−IgA)
A8 (EBNA1−IgA)
A1 (VCAp18−IgA)
A17 (EAd−IgA)
Spearman correlation
0 0.2 0.4 0.6 0.8 1