Published in final edited form as:
J Med Virol. 2013 March ; 85(3): 524–529. doi:10.1002/jmv.23498.

Characterization of ELISA Detection of Broad-spectrum Anti-Epstein-Barr Virus Antibodies Associated with Nasopharyngeal Carcinoma

Cindy Chang[1,*], **Jaap Middeldorp**[2], **Kelly J. Yu**[1,3], **Hedy Juwana**[2], **Wan-Lun Hsu**[4,5], **Pei-Jen Lou**[6], **Cheng-Ping Wang**[6], **Jen-Yang Chen**[7], **Mei-Ying Liu**[8], **Ruth M Pfeiffer**[1], **Chien-Jen Chen**[4,5], and **Allan Hildesheim**[1,*]

[1]Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Department of Health and Human Services, Rockville, MD [2]Department of Pathology, Vrije Universiteit Medical Center, Amsterdam, The Netherlands [3]Division of Cancer Prevention, National Cancer Institute, National Institutes of Health, Department of Health and Human Services, Rockville, MD [4]Graduate Institute of Epidemiology, College of Public Health, National Taiwan University, Taipei, Taiwan [5]Genomics Research Center, Academia Sinica, Taipei, Taiwan [6]Department of Otolaryngology, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan [7]National Institute of Cancer Research, National Health Research Institutes, 35, Keyan Road, Zhunan Town, Miaoli County 350, Taiwan [8]Center of General Education, National Taipei University of Nursing and Health Sciences, Taipei, Taiwan

Abstract

Epstein-Barr virus (EBV) infection is associated with undifferentiated nasopharyngeal carcinomas (NPC). A distinct seroreactivity pattern to EBV is predictive of subsequent risk of sporadic and familial nasopharyngeal carcinomas. There are currently no accepted screening tools for guiding the clinical management of individuals at high-risk for nasopharyngeal carcinomas, particularly unaffected relatives from nasopharyngeal carcinoma multiplex families. Therefore, the reproducibility of a panel of largely synthetic peptide-based anti-EBV antibody ELISAs was evaluated and their ability to distinguish nasopharyngeal carcinoma cases from controls was explored. IgG and IgA antibodies against 6 different EBV antigens (10 assays, total) were tested on sera from 97 individuals representing the full spectrum of anti-EBV seroprevalence (i.e., healthy individuals with no known EBV seroreactivity, healthy individuals with known EBV seroreactivity, and nasopharyngeal carcinoma cases). Each specimen was tested in triplicate to assess within-batch and across-batch variation, and the triplicate testing was repeated on two separate days. Reproducibility was assessed by the coefficients of variation (CV) and intraclass correlation coefficients (ICC). All markers were detectable in 17% or more of samples. For all but one marker, the overall, within-batch, and across-batch CVs were below 15%, and the ICCs were above 70% for all but three markers. Sensitivity of these markers to detect prevalent nasopharyngeal carcinomas ranged from 22–100%, and among unaffected controls, most distinguished those with and without known seropositivity. In conclusion, a large number of EBV markers can be measured reliably in serum samples using peptide-based anti-EBV ELISAs.

[*]Correspondence to: Allan Hildesheim, PhD Division of Cancer Epidemiology and Genetics National Cancer Institute, NIH, DHHS 6120 Executive Blvd., EPS 7066 Rockville, MD 20892 Phone: 301-435-3984 Fax: 301-402-0817 hildesha@mail.nih.gov.

Keywords

Epstein-Barr virus; EBNA1; VCA; IgA; nasopharyngeal carcinoma; screening

Introduction

Epstein-Barr virus (EBV) infection is closely associated with the development of nearly all undifferentiated nasopharyngeal carcinomas, a rare cancer with elevated rates in South-East Asian and Chinese populations. Nasopharyngeal carcinoma cases are known to develop a distinct anti-EBV antibody profile that is characterized by the presence of antibodies, particularly IgA antibodies against structural (viral capdid antigen or VCA) and some non-structural (early antigen or EA, Epstein-Barr nuclear anigen or EBNA, deoxyribonuclease or DNase) proteins [Henle and Henle, 1976; Baskies et al., 1979; Cheng et al., 1980; Chen et al., 1987; Fachiroh et al., 2004]. This distinct seroreactivity pattern was shown to be predictive of subsequent risk of disease in large prospective cohorts conducted in nasopharyngeal carcinoma high-risk areas [Chien et al., 2001; Cao et al., 2011]. The EBV seroreactivity pattern observed in nasopharyngeal carcinoma patients has been mapped to molecularly-defined viral proteins and peptide epitopes on these proteins, allowing for the design of more simplified, standardized assays [Dardari et al., 2001; Fachiroh et al., 2006; Paramita et al., 2007].

Individuals with a family history of nasopharyngeal carcinomas have been shown to have a much higher absolute risk of developing the disease [Friborg et al., 2005; Yu et al., 2009]. In a previous study in Taiwan, healthy individuals from 358 families with 2 or more individuals affected with nasopharyngeal carcinomas were followed for incident nasopharyngeal carcinomas. The rates of nasopharyngeal carcinomas were observed to be >10 times higher among unaffected family members from multiplex families compared to the general population (90 per 100,000 person-years vs. 7 per 100,000 person-years) [Yu et al., 2011]. Currently there are no accepted screening tools that can be applied to this high-risk population for guiding their clinical management. In a recent study, it was shown that among individuals within nasopharyngeal carcinoma multiplex families, the presence of IgA antibodies against EBNA-1 was associated with a 6.6-fold increased risk of nasopharyngeal carcinomas [Yu et al., 2011].

Taken together, previous work suggests that EBV serology screening has promise as a clinical tool to guide clinicians in the management of individuals at high-risk of nasopharyngeal carcinomas, but will require assays for EBV markers that are highly reproducible and both highly sensitive and specific for nasopharyngeal carcinomas. Therefore, the goal of this study is to define the reproducibility of a panel of largely synthetic peptide-based anti-EBV antibody ELISA tests and provide additional initial findings exploring their performance for distinguishing nasopharyngeal carcinoma cases from controls. IgA antibodies to EBV peptides previously associated with nasopharyngeal carcinoma risk were the focus and, for completeness, assays that detect IgG antibodies to these same peptides were also evaluated [Cheng et al., 2002; Chan et al., 2003; Tang et al., 2007; Liu et al., 2012].

Methods

Study Population

97 individuals selected for this study were participants in our previously described case-control [Hildesheim et al., 2001] and family studies of nasopharyngeal carcinomas in Taiwan [Yang et al., 2005]. Individuals represented the spectrum of anti-EBV

seroprevalence for which the assays would be expected to perform well. Therefore, 20 histologically confirmed nasopharyngeal carcinoma cases from the case-control study along with three groups of controls from the family study, all of whom had a family history of nasopharyngeal carcinomas in 2 or more first-degree relatives, were selected. Controls were selected from the family study because they had a wider range of seroreactivity than the controls from the case-control study and were therefore more informative for purposes of assessing assay performance [Pickard et al., 2004]. Control group 1 were 32 unaffected individuals with known seroreactivity to 2 or more of the three anti-EBV antibody tests (anti-EBNA1 IgA, anti-VCA IgA, and anti-DNase, based on previously published results); control group 2 were 25 unaffected individuals with known seroreactivity to only one of the three anti-EBV antibody test results; and control group 3 were 20 unaffected individuals expected to be EBV infected but with no seroreactivity to the three anti-EBV antibody test results. Sera from nasopharyngeal carcinoma cases were obtained from blood collected prior to the initiation of treatment. Ethical approval by an institutional review board (IRB) was obtained in both the U.S. and Taiwan. All subjects provided informed consent.

Specimen Selection and Batching

For each individual selected for study, three identical, masked aliquots of serum for testing were prepared. Two aliquots were placed randomly within one testing box and a third aliquot was placed randomly in a separate box. One sample had low quantity of serum and therefore was aliquoted to be tested only in duplicate within one box. A total of 7 testing boxes were shipped to the laboratory for testing. At the laboratory, aliquots from each box were further aliquoted onto duplicate wells of a single 96-well ELISA plate. Each plate consisted of 80 to 84 test wells, while the remaining wells on each plate were utilized for positive and negative controls as described further below. Finally, each plate for each assay was prepared and tested on two separate days (by the same technician), with a median of 16 days apart (range: 2 to 43 days). Thus, each specimen was tested in triplicate to assess within-batch and across-batch variation, and the triplicate testing was repeated on two separate days for a total of 6 test results per assay.

Anti-EBV Antibody Tests

High binding 96-well ELISA plates (Costar 9018, Thermo Fisher Scientific, Pittsburgh, Pennsylvania, U.S.A.) were coated at 4°C overnight with 100 μl per well of 50mM carbonate-bicarbonate buffer, pH 9.6 containing either 1.0 μg/ml EBV-peptides (VCA-p18, EBNA1, EAp138, EA-diffuse or EAd and Zebra) or nuclear EA extract (EA ext) in 150 μl, exactly as previously described (Table 1) [Meij et al., 1999; Fachiroh et al., 2006; Paramita et al., 2007; Asito et al., 2010]. Subsequently, wells were emptied and filled with 200 μl 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and incubated for 1 hour at 37°C, and then washed 4 times with PBS containing 0.05% Tween-20 (PBS-T). Preparation of ELISA plates was performed on each testing day using a single lot of reagents prepared for this study. Individual wells were incubated for 1 hour with 100 μl of human sera diluted 1:100 in sample buffer (PBS-T with 0.1% Triton-X, 1% BSA and 1% normal rabbit serum) at 37°C, washed 4 times with PBS-T and incubated for 1 hour at 37°C with rabbit anti-human IgG- or IgA-HRP conjugate (DAKO, Copenhagen, Denmark), diluted 1:1000 in sample buffer. After 4 washings with PBS-T, 100 μl TMB substrate solution (Sigma-Aldrich, St. Louis, Missouri, U.S.A.) was added and after 30 minutes, the reaction was stopped by adding 100 μl 1M H_2SO_4. Absorbance at 450 nm was measured in a Tecan Spectrafluor ELISA reader (software: Xfluor-4, Tecan Benelux, Männedorf, Switzerland). In each ELISA test, 2 known EBV IgG/IgA positive reference sera were tested at 1:100 in duplicate calibrators and the cut-off value (COV) for each ELISA plate was defined by calculating the mean OD450 reactivity + 3 multiplied by the SD of 4 defined EBV negative sera (1:100) tested in duplicate. Values below the COV were considered negative. Results

were reported as the mean of the duplicate absorbance value observed for each test specimen, divided by the COV.

Statistical Analysis

Results obtained from the laboratory were multiplied by 100 and log transformed for normalization. Analyses were performed on the log-transformed values. Overall positivity rates were calculated for all specimens tested, and among positives the geometric mean titer (GMT) was obtained. Coefficients of variation (CVs; overall, within plate and between plate) for each assay were computed to obtain estimates for assay variability, both overall and separately within and between testing batches. To estimate the proportion of the total assay variability attributed to variability between individuals, intraclass correlation coefficients (ICCs) for each assay was calculated using variance components analyses formulated as a linear mixed model, fitted with PROC MIXED (SAS® version 9.2, Cary, North Carolina, U.S.A.). On each testing day, ELISA plates were prepared freshly, with newly diluted antigen and conjugates, introducing variability, not encountered if industrial freeze-dried plates and reagents are used. Thus, the random effect in the mixed models included testing date, box, and repeated aliquots of the same person. Study group (defined by nasopharyngeal carcinomas and previously available EBV antibody status as specified above) was included as a fixed effect. For 3 of the assays (EA ext IgA, EA ext IgG, and EAd IgA), the models including “testing date” as a random effect did not converge, and, instead, we included “test day” (coded as 1 or 2) as a fixed effect.

Percent agreement per assay was calculated based on dichotomous test results by taking the mean of the mean agreement across all test results per individualAnalyses were stratified by study groups. For this exploratory evaluation, the percent seropositivity (and GMT among seropositives) for each assay by study group were additionally estimated.

Results

For both days, a total of 580 serum samples from 97 individuals were tested with a panel of 10 anti-EBV ELISA assays (Table 2). The percent positivity among the samples for all of the markers ranged from 17.8% to 71.0%. The GMT for the markers ranged from 65.3 to 140.0, and the GMT among seropositives ranged from 154.3 to 287.5. The percent positivity and GMT among positives were comparable between the 2 test days for each marker (data not shown).

Table 3 presents the CVs (within batch, across batch and overall) and ICCs by anti-EBV ELISA serology test. Percent agreement based on positive/negative test results was high for all 10 assays (81.9% to 90.6%). The within-batch CVs ranged from 7.0% (EA ext IgG) to 13.0% (EA ext IgA), while the across-batch CVs ranged from 2.0% (EAp138 IgA) to 10.9% (EA ext IgA) and the ICCs ranged from 42.3% (EA ext IgA) to 87.7% (VCAp18 IgA). For all assays, with the exception of the EA ext assays, CVs were comparable between experiments conducted on separate dates (p-values for date of testing > 0.05). For all but 1 marker (EA ext IgA), the overall CVs were lower than 15%, and for all but three assays (EA ext IgA, EA ext IgG, and Zebra IgA) the ICCs were greater than 70%.

When percent detection and GMTs were evaluated by study group (Table 4), detection rates and GMTs were typically observed to be highest among nasopharyngeal carcinoma cases and lower among healthy individuals with decreasing evidence of EBV seroreactivity (as defined in the methods). High sensitivity for the detection of nasopharyngeal carcinomas was observed for the EBNA IgA (99.2%), VCAp18 IgA (85.0%), EA ext IgG (100%) and Zebra IgG (85.5%) assays. High specificity (defined as 1 minus percent positive among

control group 3) was observed for the EBNA IgA (88.3%), VCAp18 IgA (80.0%), EA ext IgA (87.5%), EAp138 IgA (97.5%), EAd IgA (95.0%) and Zebra IgA (84.2%) assays.

Discussion

EBV serology has been shown to be a predictor of NPC risk for both sporadic and familial NPC [Zeng et al., 1985; Chien et al., 2001; Yu et al., 2011]. General population screening is unlikely to be practical, but there is a need for markers of risk to guide clinical management within high risk families. However, before any EBV markers can be used clinically, it is important to a) develop and characterize assays with potential to be developed further as "highly standardized" commercial tests and b) to show that these markers perform well with high sensitivity/specificity and predictive value within populations of interest. In this paper, initial steps have been taken in this direction to characterize carefully an ELISA-based set of anti-EBV antibody assays that have the potential for commercialization and standardization.

Most of the 10 assays evaluated had good reproducibility. However, IgG assays, though reproducible, are unlikely to be very useful for screening purposes since they are a good marker of exposure to the highly prevalent EBV but do not distinguish well between nasopharyngeal carcinoma cases and non-cases. In contrast, EBV IgA assays appear to provide promising serological tools for first- line nasopharyngeal carcinoma risk assessment. In particular, the EBNA1 IgA and VCA IgA assays, which use synthetic peptides, were reproducible and higher in nasopharyngeal carcinoma cases than in controls. Interestingly, the same two antigens based on different assays together had high sensitivity and specificity in a recent study in China [Liu et al., 2012]. Finally, the assays based on purified cell extracts, such as EA ext IgA, which are produced in a less standardized fashion than for synthetic peptides, had higher CVs and lower ICCs.

Limitations of this study include the small sample size and the exploratory nature of the specificity and sensitivity analysis. Strengths of this study are the ability to select samples taken from individuals with 4 distinct anti-EBV profiles; that samples were collected, processed and stored based on a standardized protocol; and that 10 anti-EBV markers were comprehensively evaluated.

In conclusion, most of the anti-EBV markers are broadly reproducible and can be used for large-scale screening studies that may eventually lead to the clinical management of individuals at high-risk of nasopharyngeal carcinomas. Future studies should evaluate the performance of these assays (and combination of assays), particularly IgA assays, for predicting risk of nasopharyngeal carcinomas within high-risk individuals.

Acknowledgments

This research was supported by the Intramural Research Program of the National Cancer Institute, National Institutes of Health.

References

Asito AS, Piriou E, Odada PS, Fiore N, Middeldorp JM, Long C, Dutta S, Lanar DE, Jura WG, Ouma C, Otieno JA, Moormann AM, Rochford R. Elevated anti-Zta IgG levels and EBV viral load are associated with site of tumor presentation in endemic Burkitt's lymphoma patients: a case control study. Infect Agent Cancer. 2010; 5:13. [PubMed: 20667138]

Baskies AM, Chretien PB, Yang C, Wolf GT, Makuch RW, Tu S, Hsu M, Lynn T, Yang H, Weiss JF, Spiegel HE. Serum glycoproteins and immunoglobulins in nasopharyngeal carcinoma: correlations with Epstein-Barr virus associated antibodies and clinical tumor stage. Am J Surg. 1979; 138:478–488. [PubMed: 225960]

Cao SM, Liu Z, Jia WH, Huang QH, Liu Q, Guo X, Huang TB, Ye W, Hong MH. Fluctuations of epstein-barr virus serological antibodies and risk for nasopharyngeal carcinoma: a prospective screening study with a 20-year follow-up. PLoS One. 2011; 6:e19100. [PubMed: 21544243]

Chan KH, Gu YL, Ng F, Ng PS, Seto WH, Sham JS, Chua D, Wei W, Chen YL, Luk W, Zong YS, Ng MH. EBV specific antibody-based and DNA-based assays in serologic diagnosis of nasopharyngeal carcinoma. Int J Cancer. 2003; 105:706–709. [PubMed: 12740922]

Chen JY, Chen CJ, Liu MY, Cho SM, Hsu MM, Lynn TC, Shieh T, Tu SM, Lee HH, Kuo SL, et al. Antibodies to Epstein-Barr virus-specific DNase in patients with nasopharyngeal carcinoma and control groups. J Med Virol. 1987; 23:11–21. [PubMed: 2824675]

Cheng WM, Chan KH, Chen HL, Luo RX, Ng SP, Luk W, Zheng BJ, Ji MF, Liang JS, Sham JS, Wang DK, Zong YS, Ng MH. Assessing the risk of nasopharyngeal carcinoma on the basis of EBV antibody spectrum. Int J Cancer. 2002; 97:489–492. [PubMed: 11802211]

Cheng YC, Chen JY, Glaser R, Henle W. Frequency and levels of antibodies to Epstein-Barr virus-specific DNase are elevated in patients with nasopharyngeal carcinoma. Proc Natl Acad Sci U S A. 1980; 77:6162–6165. [PubMed: 6255477]

Chien YC, Chen JY, Liu MY, Yang HI, Hsu MM, Chen CJ, Yang CS. Serologic markers of Epstein-Barr virus infection and nasopharyngeal carcinoma in Taiwanese men. N Engl J Med. 2001; 345:1877–1882. [PubMed: 11756578]

Dardari R, Hinderer W, Lang D, Benider A, El Gueddari B, Joab I, Benslimane A, Khyatti M. Antibody responses to recombinant Epstein-Barr virus antigens in nasopharyngeal carcinoma patients: complementary test of ZEBRA protein and early antigens p54 and p138. J Clin Microbiol. 2001; 39:3164–3170. [PubMed: 11526145]

Fachiroh J, Paramita DK, Hariwiyanto B, Harijadi A, Dahlia HL, Indrasari SR, Kusumo H, Zeng YS, Schouten T, Mubarika S, Middeldorp JM. Single-assay combination of Epstein-Barr Virus (EBV) EBNA1- and viral capsid antigen-p18-derived synthetic peptides for measuring anti-EBV immunoglobulin G (IgG) and IgA antibody levels in sera from nasopharyngeal carcinoma patients: options for field screening. J Clin Microbiol. 2006; 44:1459–1467. [PubMed: 16597877]

Fachiroh J, Schouten T, Hariwiyanto B, Paramita DK, Harijadi A, Haryana SM, Ng MH, Middeldorp JM. Molecular diversity of Epstein-Barr virus IgG and IgA antibody responses in nasopharyngeal carcinoma: a comparison of Indonesian, Chinese, and European subjects. J Infect Dis. 2004; 190:53–62. [PubMed: 15195243]

Friborg J, Wohlfahrt J, Koch A, Storm H, Olsen OR, Melbye M. Cancer susceptibility in nasopharyngeal carcinoma families--a population-based cohort study. Cancer Res. 2005; 65:8567–8572. [PubMed: 16166338]

Henle G, Henle W. Epstein-Barr virus-specific IgA serum antibodies as an outstanding feature of nasopharyngeal carcinoma. Int J Cancer. 1976; 17:1–7. [PubMed: 175020]

Hildesheim A, Dosemeci M, Chan CC, Chen CJ, Cheng YJ, Hsu MM, Chen IH, Mittl BF, Sun B, Levine PH, Chen JY, Brinton LA, Yang CS. Occupational exposure to wood, formaldehyde, and solvents and risk of nasopharyngeal carcinoma. Cancer Epidemiol Biomarkers Prev. 2001; 10:1145–1153. [PubMed: 11700262]

Liu Y, Huang Q, Liu W, Liu Q, Jia W, Chang E, Chen F, Liu Z, Guo X, Mo H, Chen J, Rao D, Ye W, Cao S, Hong M. Establishment of VCA and EBNA1 IgA-based combination by enzyme-linked immunosorbent assay as preferred screening method for nasopharyngeal carcinoma: a two-stage design with a preliminary performance study and a mass screening in southern China. Int J Cancer. 2012; 131:406–416. [PubMed: 21866545]

Meij P, Vervoort MB, Aarbiou J, van Dissel P, Brink A, Bloemena E, Meijer CJ, Middeldorp JM. Restricted low-level human antibody responses against Epstein-Barr virus (EBV)-encoded latent membrane protein 1 in a subgroup of patients with EBV-associated diseases. J Infect Dis. 1999; 179:1108–1115. [PubMed: 10191211]

Paramita DK, Fachiroh J, Artama WT, van Benthem E, Haryana SM, Middeldorp JM. Native early antigen of Epstein-Barr virus, a promising antigen for diagnosis of nasopharyngeal carcinoma. J Med Virol. 2007; 79:1710–1721. [PubMed: 17854043]

Pickard A, Chen CJ, Diehl SR, Liu MY, Cheng YJ, Hsu WL, Sun B, Hsu MM, Chen IH, Chen JY, Yang CS, Mittl BL, Chou SP, Ruggles DD, Goldstein AM, Hildesheim A. Epstein-Barr virus

seroreactivity among unaffected individuals within high-risk nasopharyngeal carcinoma families in Taiwan. Int J Cancer. 2004; 111:117–123. [PubMed: 15185352]

Tang JW, Rohwader E, Chu IM, Tsang RK, Steinhagen K, Yeung AC, To KF, Chan PK. Evaluation of Epstein-Barr virus antigen-based immunoassays for serological diagnosis of nasopharyngeal carcinoma. J Clin Virol. 2007; 40:284–288. [PubMed: 17977062]

Yang XR, Diehl S, Pfeiffer R, Chen CJ, Hsu WL, Dosemeci M, Cheng YJ, Sun B, Goldstein AM, Hildesheim A. Evaluation of risk factors for nasopharyngeal carcinoma in high-risk nasopharyngeal carcinoma families in Taiwan. Cancer Epidemiol Biomarkers Prev. 2005; 14:900–905. [PubMed: 15826929]

Yu KJ, Hsu WL, Chiang CJ, Cheng YJ, Pfeiffer RM, Diehl SR, Goldstein AM, Gravitt PE, Chen CJ, Hildesheim A. Cancer patterns in nasopharyngeal carcinoma multiplex families in Taiwan. Int J Cancer. 2009; 124:1622–1625. [PubMed: 19065653]

Yu KJ, Hsu WL, Pfeiffer RM, Chiang CJ, Wang CP, Lou PJ, Cheng YJ, Gravitt P, Diehl SR, Goldstein AM, Chen CJ, Hildesheim A. Prognostic Utility of Anti-EBV Antibody Testing for Defining NPC Risk among Individuals from High-Risk NPC Families. Clin Cancer Res. 2011; 17:1906–1914. [PubMed: 21447725]

Zeng Y, Zhang LG, Wu YC, Huang YS, Huang NQ, Li JY, Wang YB, Jiang MK, Fang Z, Meng NN. Prospective studies on nasopharyngeal carcinoma in Epstein-Barr virus IgA/VCA antibody-positive persons in Wuzhou City, China. Int J Cancer. 1985; 36:545–547. [PubMed: 4055129]

Table 1

Sequences of Target Peptides Used in Anti-EBV ELISAs

Peptide EBNA (BKRF1):
"PPRRPPPGRRPFFHPVGEADYFEYHQEDGEPDVPPGAIEQGPADDPGEGPSTGPRG"[a]
Peptide VCA-p18 (BFRF3):
"STAVAQSATPSVSSSISSLRAATSGATAAAAVDTGSGGGGQPHDTAPRGARKKQ"[a]
Peptide EAd-p47 (BMRF1):
"TVSPSPSPPPPPRTPTWESHSSNTALERPLAVQLARKRTSSEARQ"[a]
Peptide EAd-p138 (BALF2): "KSVRVPLYDKEVFPEGVPQLRQFYNSDLSLGIDAEGKL" +"KTGTNGPGNYAVEHLVYAASFSPNLLARYAYYLQF"[a]
Peptide Zebra (BZLF1): "MMDPNSTSEDVKFTPDPYQVPFVQAFDQATRVYQDLGGPSQAPLPCV"

[a] patent protected and proprietary to Cyto-Barr B.V. (Zuidhorn, Netherlands)

Table 2

Percent Detectability and GMT by Anti-EBV ELISA Serology Test - EBV Serology Reproducibility Study

Assay	# Individuals/Specimens Tested	% Positive[a]	Overall GMT	GMT among Positives[a]
EBNA IgA	97/580	48.6%	127.2	287.5
VCAp18 IgA	97/580	58.6%	135.0	225.9
EA ext IgA	97/580	31.2%	84.0	169.9
EAp138 IgA	97/580	18.1%	67.7	154.6
EAd IgA	97/580	17.8%	69.8	195.1
Zebra IgA	97/580	17.4%	65.3	154.3
EA ext IgG	97/580	71.0%	140.0	176.6
EAp138 IgG	97/580	41.6%	94.6	173.1
EAd IgG	97/580	33.6%	82.2	207.0
Zebra IgG	97/580	67.6%	139.3	191.2

[a]Statistics are based on specimen not individual-level results

Table 3

Assay Performance - Coefficients and Variantion and Interclass Correlation Coefficients by Anti-EBV ELISA Serology Test - EBV Serology Reproducibility Study[a]

Assay	# Individuals Tested	% Agreement[c]	Within Batch	Across Batch	Overall	
			CV	CV	CV	ICC
EBNA IgA	97	89.7%	11.0	6.8	12.9	78.1
VCAp18 IgA	97	88.7%	7.3	5.8	9.4	87.7
EA ext IgA[b]	97	87.6%	13.0	10.9	17.0	42.3
EAp138 IgA	97	87.6%	10.0	2.0	10.2	73.3
EAd IgA[b]	97	90.7%	10.6	5.1	11.8	73.9
Zebra IgA	97	89.7%	9.1	8.6	12.5	67.1
EA ext IgG[b]	97	82.1%	7.0	8.2	10.8	65.3
EAp138 IgG	97	81.1%	10.5	5.3	11.8	74.6
EAd IgG	97	90.0%	12.6	6.6	14.2	71.3
Zebra IgG	97	85.2%	8.5	4.5	9.6	82.0

[a]Random effect in the mixed models included testing date, box, and repeated aliquots of the same person; study group (defined by NPC and EBV antibody status) was included as a fixed effect.

[b]Models including “testing date” as a random effect did not converge; “test day” (coded as 1 or 2) was included as a fixed effect instead.

[c]Agreement based on dichotomous results of each assay as described in the Methods

Table 4

Ability of Assays to Distinguish NPC Cases from Non-Cases by Anti-EBV ELISA Serology Test - EBV Serology Reproducibility Study

Assay	NPC Cases (N=20)	% Positivity (GMT among Positives)			P-value
		Control Group 1 (2+)[a] (N=32)	Control Group 2 (1)[a] (N=25)	Control Group 3 (0)[a] (N=20)	
EBNA IgA	99.2% (420.5)	52.6% (291.5)	32.7% (140.3)	11.7% (126.7)	<.0001[b]
VCAp18 IgA	85.0% (228.9)	72.1% (260.8)	51.3% (207.6)	20.0% (123.1)	<.0001[b]
EA ext IgA	77.5% (191.0)	24.7% (161.0)	17.3% (143.4)	12.5% (130.8)	<.0001[c]
EAp138 IgA	43.3% (178.6)	16.3% (149.8)	12.7% (115.7)	2.5% (110.3)	<.0001[b]
EAd IgA	50.0% (256.9)	12.6% (138.1)	8.7% (123.8)	5.0% (132.3)	<.0001[c]
Zebra IgA	22.5% (220.9)	18.4% (128.9)	13.3% (143.4)	15.8% (139.3)	0.0026[b]
EA ext IgG	100% (264.2)	73.2% (161.8)	64.7% (143.9)	46.7% (131.9)	<.0001[c]
EAp138 IgG	66.7% (236.9)	36.3% (155.0)	36.0% (142.4)	31.7% (144.5)	<.0001[b]
EAd IgG	76.7% (268.1)	21.1% (193.0)	25.3% (150.2)	20.8% (145.4)	<.0001[b]
Zebra IgG	85.5% (216.8)	63.7% (190.2)	66.7% (174.5)	56.7% (182.2)	0.0004[b]

[a] Controls grouped based on the number of positive results out of three anti-EBV antibody tests as described in the Methods

[b] P-value for study group as fixed effect in mixed models also including as random effects, testing date, box, and repeated aliquots of the same person

[c] Models including “testing date” as a random effect did not converge; “test day” (coded as 1 or 2) was included as a fixed effect instead.