Supplementary Materials

Assay A1 (VCAp18-IgA; research assay)

ELISA plates were coated at 4°C overnight with 100 µl per well of 50 mM carbonate–bicarbonate buffer, pH 9.6 containing 1.0 µg/ml EBV-peptides (VCA-p18), as described in **Supplementary Table 1**. Subsequently, wells were emptied and filled with 200 µl 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and incubated for 1 hr at 37°C, and then washed four times with PBS containing 0.05% Tween-20 (PBS-T). Preparation of ELISA plates was performed using a single lot of reagents prepared for this study. Individual wells were incubated for 1 hr 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 four times with PBS-T and incubated for 1 hr at 37°C with rabbit anti-human IgG- or IgA-HRP conjugate (DAKO, Copenhagen, Denmark), diluted 1:1,000 in sample buffer. After four washings with PBS-T, 100 µl TMB substrate solution (Sigma-Aldrich, St. Louis, MO) was added and after 30 min, the reaction was stopped by adding 100 µl 1 M H2SO4. Absorbance at 450 nm was measured in a Tecan Spectrafluor ELISA reader (software: Xfluor-4, Tecan Benelux, Männedorf, Switzerland). In each ELISA test, two 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.

Assay A2.1 (VCA-IgA; commercial assay)

Individual wells were added with 100 µl of test sera including in-house positive control diluted 1:100 in 1X sample buffer and the positive and/or negative controls in kit. Then the ELISA plates were incubated on Shaker in room temperature (18-25℃) for 30 minutes, washed three times with 1X Wash Buffer and incubated in room temperature (18-25℃) for 30 minutes with rabbit anti-human IgA-HRP conjugate used directly. After three washings with 1X Wash Buffer, 100 µl TMB substrate solution was added and after 15 min, the reaction was stopped by adding 100 µl 0.5 M H2SO4. Absorbance at 450 nm was measured in a Thermo Multiskan GO ELISA reader (software: SkanIt Software Version 3.2.0.35). In each ELISA test, one well of blank control, one well of negative controls and three known EBV IgG/IgA positive reference sera including two wells of positive controls, two wells of in-house positive controls and in duplicate calibrators were tested and the relative antibody level (rOD) of VCA/IgA for each sample was defined by calculating the OD of (sample - Blank Control)/ the average OD of (calibrators - 2Blank Control).

Assay A2.2 (VCA-IgA; commercial assay)

The laboratory testing was similar to Assay 2.1, except for the fact that no in-house positive control samples were added in the testing. Therefore, the calibration was only performed based on calibrator, positive and negative control samples provided in the kit.

Assay A3 (VCA-IgA; commercial assay)

For the ELISA assay, equilibrated the detection kit to room temperature before use. Individual wells were incubated for 30 min with 5 µl of human sera/plasma and 100 µl sample diluent at 37°C, washed five times with washing buffer (PBS-T) and incubated for 30 min at 37°C with 100µl anti-human IgA-HRP

conjugate. After five washings with PBS-T, 50 µl substrate solution A and 50 µl substrate solution B were added respectively and after 15 min preventing from light at 37°C, the reaction was stopped by adding 50 µl stopping solution (1 mol/l H2SO4).

Absorbance at 450 nm was measured using Multiskan™ FC Microplate Photometer (Thermo Scientific) and reported as OD from duplicates. In each ELISA test, known EBV IgA positive control sera and negative control sera were tested in duplicate and the cut-off value (COV) for each ELISA plate was defined by calculating the mean OD450 positive control*20%. The mean of the duplicate absorbance value observed for each test specimen below the COV was considered negative, equal to or greater than the COV were considered positive.

Assay A4 (VCAp18-IgA; research assay)

Antibodies against the EBV antigen (**Supplementary Table 1**) were measured using Multiplex Serology as described previously (1, 2). This high-throughput technology allows the measurement of antibodies against all included EBV-proteins simultaneously.

GST-EBV antigen-tag fusion proteins (VCA-p18, EBNA1-peptide, EBNA1-truncated, EAd and Zta; shown in **Supplementary Table 1)** and one protein consisting of GST and tag alone (GST-tag), were expressed in *E. coli* strain BL21. The antigens were *in situ* affinity-purified from bacterial lysates through binding to glutathione casein-coated fluorescence labelled polystyrene beads. Each fusion protein was loaded onto a spectrally distinct bead set and all bead sets were subsequently combined into one bead mix.

Sera were pre-incubated for one hour at serum dilution 1:50 in PBS containing 1 mg/mL casein, 2 mg/mL GST-tag lysate to block antibodies directed against residual bacterial proteins and GST-tag, 0.5% polyvinylalcohol, and 0.8% polyvinylpyrrolidone to suppress unspecific binding of antibodies to the beads themselves.

The diluted sera were incubated for one hour with the same volume of bead mix, resulting in a final serum dilution of 1:100. The beads were washed three times with PBS containing 1 mg/mL casein, incubated with biotinylated goat-anti-human IgA secondary antibody (#109-065-011, Jackson Immunoresearch) for one hour, washed three times with PBS containing 1 mg/mL casein and incubated with reporter conjugate streptavidin-R phycoerythrin (1:750, PE-Streptavidin Conjugate, MOSS Inc.) for 30 minutes.

A Luminex analyzer was used to identify the internal color of the individual beads and to quantify their reporter fluorescence (expressed as median fluorescence intensity (MFI) of at least 100 beads per set per serum). The GST-tag fusion protein served for background determination.

Quality control sera with known reactivity for every antigen were included as positive standards on each plate. Auto-fluorescence of each bead set and background reactions resulting from binding of secondary reagents to the antigen-loaded beads were determined in one well per plate without human serum. Mean background values were subtracted from the raw MFI values. Antigen-specific reactivity was determined by subsequent subtraction of the MFI of GST-tag from the MFI of the specific antigen. The antigen-specific cut-offs to determine seropositivity are displayed in **Supplementary Table 1**.

Assay A5 (VCAp18-IgA; research assay)

Serum and plasma IgA antibodies against synthetic peptides covering immunodominant epitopes of the viral capsid antigen P18 (VCA), were measured using a multiplexed bead-based assay as previously

described (3), at a 1:200 serum dilution. Results were recorded as background-subtracted, median fluorescent intensity levels (MFI) from a Bio-Rad 200 multiplex reader.

Assay A6 (VCAp18-IgG; research assay)

Refer to Assay A5.

Assay A7 (VCAp18-IgG/IgA/IgM; research assay)

The general assay procedure was described for Assay A4. Thus, only steps deviating in performance are described in this section.

Sera were pre-incubated for one hour at serum dilution 1:5000. Preincubation-buffer additionally contained 2.5% CBS-K. Serum dilutions were incubated for one hour with the same volume of mixed bead sets, resulting in a final serum dilution of 1:10,000. As secondary antibody a biotinylated goat-anti-human triple (IgG/IgM/IgA) antibody (#109-065-064, Jackson Immunoresearch) was used.

Assay A8 (EBNA1-IgA; research assay)

Refer to Assay A1.

Assay 9.1 (EBNA1-IgA; commercial assay)

Individual wells were added with 100 µl of test sera including in-house positive control diluted 1:100 in 1X sample buffer and the positive controls and/or negative controls in kit. Then the ELISA plates were incubated in 37℃ for 60 min, washed five times with 1X Wash Buffer and in 37℃ for 60 min with 1X rabbit anti-human IgA-HRP conjugate. After five washings with 1X Wash Buffer, 100 µl TMB substrate solution (BufferA/B) was added and after 15 min, the reaction was stopped by adding 100 µl 1M H_2SO_4. Absorbance at 450 nm was measured in a Thermo Multiskan GO ELISA reader (software: SkanIt Software Version 3.2.0.35). In each ELISA test, one well of blank control, one well of negative control and three known EBV IgG/IgA positive reference sera including two wells of positive controls, two wells of in-house positive controls and in duplicate calibrators were tested and the relative antibody level (rOD) of EBNA1/IgA for each sample was defined by calculating the OD of (sample - Blank Control)/ the average OD of (calibrators – 2 blank controls).

Assay 9.2 (EBNA1-IgA; commercial assay)

Refer to Assay A3.

Assay 10 (EBNA1-IgA; research assay)

Refer to Assay A4.

Assay 11 (EBNA1-IgA; research assay)

Refer to Assay A4.

Assay 12 (EBNA1-IgA; research assay)

Refer to Assay A5.

Assay 13 (EBNA1-IgG; research assay)

Refer to Assay A5.

Assay 14 (EBNA1 peptide-IgG/IgA/IgM; research assay)

Refer to Assay A7.

Assay 15 (EBNA1 truncated-IgG/IgA/IgM; research assay)

Refer to Assay A7.

Assay 16 (EAd-IgA; research assay)

Refer to Assay A4.

Assay 17 (EAd-IgA; research assay)

Refer to Assay A5.

Assay 18 (EAd-IgG; research assay)

Refer to Assay A5.

Assay 20 (Zta-IgA; research assay)

Refer to Assay A4.

Assay 21 (Zta-IgA; commercial assay)

Refer to Assay A3.

Assay 22 (Zta-IgG/IgA/IgM; research assay)

Refer to Assay A7.

Assay 23 (EA-EBNA1-IgA; commercial assay)

All reagents and samples stayed in room temperature (21-25°C). A total of 1x Washing Solution by diluting 20x concentrated Washing Solution with reagent grade water were prepared and mixed with diluted serum samples with Serum Diluents (1 ml Serum Diluent + 10 µl serum). Individual wells were added with 100 µl of blank, negative controls, positive controls and diluted serum samples, and incubated at 37°C for 60 minutes. Individual wells were washed 3 times with 280-300 µl Washing Solution. After washings, 100 µl conjugate was added and incubated for 30 minutes at 37°C. Individual wells were washed 3 times with 280-300 µl Washing Solution and added with 100 µl of Substrate (TMB). After incubation at 37°C for 10 minutes, 100 µl stop solution (1N HCl) was added to wells. Assay plate then was read at 450 nm using 650 nm as reference. In each ELISA test, a standard curve was draw using OD of 3 positive controls and negative control and corresponding EU/ml and the cut-off value (COV) for each ELISA plate was defined by OD value corresponding to 8 EU/ml.

Assay 24 (EA-EBNA1-IgA; commercial assay)

A total of 1x Washing Solution by diluting 20x concentrated Washing Solution with reagent grade water were prepared and mixed with diluted serum samples with Serum Diluents (1 ml Serum Diluent + 5 µl serum). Individual wells were added with 100 µl of blank, negative controls, positive controls and

diluted serum samples, and incubated at 37°C for 60 minutes. Individual wells were washed 3 times with 300 µl Washing Solution. After washings, 100 µl conjugate was added and incubated for 30 minutes at 37°C. Individual wells were washed 3 times with 300 µl Washing Solution and added with 100 µl of Substrate (TMB). After incubation at 37°C for 15 minutes, 100 µl stop solution (1N HCl) was added to wells. Assay plate then was read at 450 nm using 650 nm as reference. In each ELISA test, a standard curve was draw using OD of 3 positive controls and negative control and corresponding EU/ml and the cut-off value (COV) for each ELISA plate was defined by OD value of defined EBV negative sera x 4.5, corresponding to 6 EU/ml.

Reference

1. Waterboer T, Sehr P, Michael KM, Franceschi S, Nieland JD, Joos TO, Templin MF, Pawlita M. 2005. Multiplex human papillomavirus serology based on in situ-purified glutathione s-transferase fusion proteins. Clin Chem 51:1845-53.
2. Brenner N, Mentzer AJ, Butt J, Michel A, Prager K, Brozy J, Weissbrich B, Aiello AE, Meier HCS, Breuer J, Almond R, Allen N, Pawlita M, Waterboer T. 2018. Validation of Multiplex Serology detecting human herpesviruses 1-5. PLoS One 13:e0209379.
3. Labo N, Miley W, Marshall V, Gillette W, Esposito D, Bess M, Turano A, Uldrick T, Polizzotto MN, Wyvill KM, Bagni R, Yarchoan R, Whitby D. 2014. Heterogeneity and breadth of host antibody response to KSHV infection demonstrated by systematic analysis of the KSHV proteome. PLoS Pathog 10:e1004046.