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Short communication

On optimizing the blocking step of indirect enzyme-linked immunosorbent assay for Epstein-Barr virus serology

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ABSTRACT

Background: Because blocking agent occupies most binding surface of a solid phase, its ability to prevent nonspecific binding determines the signal-to-noise ratio (SNR) and reliability of an enzyme-linked immunosorbent assay (ELISA).
Methods: We demonstrate a stepwise approach to seek a compatible blocking buffer for indirect ELISA, via a case–control study ($n = 176$) of Epstein-Barr virus (EBV)-associated nasopharyngeal carcinoma (NPC).
Results: Regardless of case–control status, we found that synthetic polymer blocking agents, mainly Ficoll and poly(vinyl alcohol) (PVA) were able to provide homogeneous backgrounds among samples, as opposed to commonly used blocking agents, notably nonfat dry milk (NFDM). The SNRs for NPC samples that correspond to blocking using PVA were approximately 3-fold, on average, higher than those blocking using NFDM. Both intra- and inter-assay precisions of PVA-based assays were <14%.
Conclusion: A blocking agent of choice should have tolerable sample backgrounds from both cases and controls to ensure the reliability of an immunoassay.

1. Introduction

For indirect enzyme-linked immunosorbent assay (ELISA), the remaining binding surface of a solid phase after antigen immobilization must be shielded from primary and secondary antibodies to avoid background or noise. In the present study, however, we found that backgrounds were varied among assayed samples even in the absence of immobilized antigen. It appeared to be due to the blocking buffer used. High variability of sample backgrounds may lead to false-positive results and poor signal-to-noise ratio (SNR). Therefore, we propose a stepwise approach to choose the best blocking agent, to optimize its blocking condition and to apply the best blocking step to indirect ELISA, through a case–control study of Epstein-Barr virus (EBV)-associated nasopharyngeal carcinoma (NPC).

We became interested in this ELISA serology because the circulating antibodies especially anti-EBV antibodies and autoantibodies against tumor-associated proteins in NPC patients are relatively higher than normal subjects [1,2]. Nevertheless, >90% of world population is infected by EBV. They may remain free of cancers provided the virus is still under control of the host immune system. It is, therefore, necessary to improve the SNR of the serological assay. With these concerns in mind, we aim to obtain homogeneous sample backgrounds by a simple, cheap yet effective blocking step for routine ELISA serology.

2. Materials and methods

2.1. Sample collection

Ethical approval for this study was granted by the Medical Ethics Committee of University Malaya Medical Centre. All biological samples were treated as potentially infectious materials. Peripheral blood was obtained from the volunteers with written consents. Plasma was saved and kept at −20 °C. In the stepwise approach, samples were randomly drawn from the collection. Samples from 88 NPC and 88 normal subjects were eventually assayed.

2.2. Comparing the effectiveness of different blocking buffers

The effectiveness of two major groups of blocking agents, namely (i) commonly used blocking agents, i.e., fatty acid free bovine serum albumin (BSA) (Sigma-Aldrich Co., St. Louis, MO), nonfat dry milk

Abbreviations: ELISA, enzyme-linked immunosorbent assay; SNR, signal-to-noise ratio; EBV, Epstein-Barr virus; NPC, nasopharyngeal carcinoma; BSA, bovine serum albumin; NFDM, nonfat dry milk; PVA, poly(vinyl alcohol); PVP, poly(vinyl pyrrolidone); PBS, phosphate buffered saline; IgG, immunoglobulin G; ZEBRA, Z-encoded broadly reactive activator; VCA, viral capsid antigen; SC2, substrate control 2; SC1, substrate control 1; CC, conjugate control; C++, strong positive control; C+, moderately positive control; C−, negative control; ln(PP), percentage positivity on a natural logarithmic scale; A_{630}, absorbance at 630 nm; PVDF, poly(vinyl difluoride).

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(NFDM) (KPL, Inc., Gaithersburg, MD) and Hammarsten grade casein (Merck KGaA, Darmstadt, Germany); and (ii) synthetic polymers, i.e., Ficoll PM400 (Sigma-Aldrich Co., St. Louis, MO), fully hydrolyzed poly(vinyl alcohol) (PVA) (Merck KGaA, Darmstadt, Germany) and poly(vinyl pyrrolidone) (PVP) (Sigma-Aldrich Co., St. Louis, MO) were examined [3–6]. Approximate molecular weights for Ficoll (400,000), PVA (15,000) and PVP (10,000) are indicated in parentheses.

The synthetic polymers were prepared in ELISA compatible diluents, i.e., 1×phosphate buffered saline (1×PBS: 137 mmol/l NaCl, 2.7 mmol/l KCl, 10 mmol/l Na_2HPO_4, 1.76 mmol/l KH_2PO_4, pH 7.4) and coating buffer (15 mmol/l Na_2CO_3, 35 mmol/l $NaHCO_3$, pH 9.6) [5]. BSA and casein were prepared by standard protocols, using 1×PBS and alkaline hydrolysis, respectively [7]; 2% (w/v) NFDM stock solution was diluted at 1:20 in sterile water, according to the manufacturer's recommendation. Except NFDM, the concentration used for all blocking buffers was 4% (w/v), because most blocking agents were claimed to be effective within 5% (w/v) in ELISA studies.

The routine serological assays (see Experimental section of the supplementary data), namely Z-encoded broadly reactive activator (ZEBRA)/IgG and viral capsid antigen (VCA) p18/IgG, were modified in order to estimate sample backgrounds. All empty wells of Maxisorp poly(styrene) microplates (Nunc, Thermo Fisher Scientific Inc., Rochester, NY) were directly coated with blocking agents, except for wells assigned to substrate control 2 (SC2) (Table S1B) [8]. Thus samples from 12 case–control pairs were assayed in the absence of immobilized antigen. Full assay controls here consist of substrate control 1 (SC1), SC2, conjugate control (CC), strong positive (C++), moderately positive (C+) and negative (C−) controls (Table S1B) [8].

It should be noted that only coated blocking agent and buffer residuals remain in SC1 prior to the addition of substrate solution, hence SC1 serves to validate the condition of quantitative substrate system. By contrast, only buffer residuals remain in SC2 prior to the addition of substrate solution. Therefore, the reading difference between SC1 and SC2 serves to monitor the non-enzymatic reactions between a blocking layer and the components of substrate system, if any. CC serves to monitor the nonspecific binding of conjugate to the solid phase upon blocking. C++, C+ and C− were predetermined by EBV-specific immunoblotting and ELISA in our laboratory.

2.3. Grid experiments

Grid experiments were performed to optimize the blocking conditions of PVA in the absence of immobilized antigen (Fig. S2A). The kosmotropes and pHs of PVA solutions were given by the diluents used, i.e., 1×PBS and coating buffer. PVA blocking buffers were serially diluted from 4% to 0.25% (w/v), to include both 1% and 0.5% (w/v) that were showed to be effective in other immunoassays [3–5]. The blocking steps were performed accordingly, under three common incubation conditions of ELISA, (i) static incubation for overnight at 4 °C, and shaking incubation for 2 h at (ii) 37 °C and (iii) room temperature [5]. Plasma pools of NPC ($n=4$) and normal ($n=4$) subjects were assayed at 1:100, 1:400 and 1:1600.

2.4. Comparing the SNRs of serological assays

To verify the results from Sections 2.2 and 2.3, sample backgrounds of additional 72 case–control pairs were checked in the absence of immobilized antigen. Blocking using (i) NFDM at 1:20 in sterile water (manufacturer's recommendation), and 4% (w/v) PVA in (ii) 1×PBS or (iii) coating buffer for 2 h at room temperature was performed accordingly.

These samples were also assayed with immobilized antigen (ZEBRA), using the three blocking steps as above. SNRs of NPC samples from the 72 case–control pairs were estimated. The signals of assayed samples were recorded from solid phases that coated with antigen and blocked with blocking buffers. The noises of assayed samples (sample backgrounds) were estimated from solid phases that blocked with blocking buffers, but not coated with antigen.

2.5. Checkerboard titrations and reproducibility of serological assays

The compatibility of PVA blocking buffer (4% (w/v) in coating buffer) with ZEBRA/IgG and VCA p18/IgG assays was examined by checkerboard titrations. C++, C+ and C− assay controls were used as primary antibodies because they could represent strong positive, moderately positive, and negative plasma samples.

The reproducibility of the PVA-based ELISAs was validated by quadruplicates of full assay controls (Table S1A) across 10 routine runs on different days.

3. Results and discussion

BSA, NFDM and casein are conventional, widely used blocking agents. Their blocking effectiveness was therefore concerned in this study. On the other hand, we selected the synthetic polymers, namely Ficoll, PVA and PVP as blocking agents [3–6], based on their high affinity toward poly(styrene) surfaces but lack of affinity toward biomolecules [9–11]. In addition, we chose low molecular weight PVA (15,000) and PVP (10,000) to achieve good surface coverage [10], meanwhile avoiding steric hindrance in the subsequent immunodetection steps. With different building blocks from proteins—hence no antigenic determinant—these synthetic polymers should not interfere with the immunoassay.

The ELISA data, i.e., sample backgrounds (in the absence of immobilized antigen) obtained from Section 2.2 were expressed as percentage positivity [12] on a natural logarithmic scale, ln(PP) according to the following equation:

$$\ln(\mathrm{PP}) = \ln[(\text{absorbance at } 630\,\text{nm}, A_{630} \text{ of a sample}/A_{630} \text{ of C++}) \times 100] \quad (1)$$

It leads us to spot the deviation of background of a sample from C++ in a normalized manner (Fig. 1; see Fig. S1 for raw absorbance values of sample backgrounds). From the comparison of normalized data, we noticed that the distinct patterns of sample backgrounds were attributed to the blocking buffers used. It should be emphasized that a blocking agent is suitable for ELISA serology only if sample backgrounds from both cases and controls are homogeneous (close to each other) and sufficiently low to avoid false-positive signals. However, high backgrounds (widely spread data points from C++) were observed for both groups of samples when using NFDM as blocking agent. Unlike commonly used blocking agents, Ficoll (in both 1×PBS and coating buffers) and PVA (in coating buffer) provided relatively low and narrow spreads of sample backgrounds from C++.

The blocking ability of PVA was further investigated because it is clinically proven to be non-immunogenic [13] and 30-fold cheaper than Ficoll. Because the assembly of PVA chains on a solid phase may be influenced by polymer concentration, composition of kosmotrope(s), temperature [10,14] and pH, we optimized the blocking steps in grid experiments (see Section 2.3; Fig. S2A).

From the grid experiments, 4% (w/v) PVA provided the most effective blocking, viz., the lowest backgrounds across different dilutions of plasma pools from both NPC ($n=4$) and normal ($n=4$) subjects (Fig. S2B). For polymer concentrations below 2% (w/v), backgrounds that correspond to PVA layers formed in 1×PBS were relatively lower than those formed in coating buffer. It may be due to higher concentrations of kosmotropes (especially NaCl) in 1×PBS, which could promote the adsorption of PVA [14]. The kosmotropes could stabilize water structure and exhibit "salting out" effect by strengthening the hydrophobic interactions between PVA and poly(styrene) surface. Therefore, available surface on the solid phase could be