

Optimisation of Indirect ELISA by Comparison of Different Antigen Preparations for Detection of Antibodies Against Schmallenberg Virus ^{[1] [2]}

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Abstract

Schmallenberg virus (SBV) infection, discovered in 2011, was reported in Europe including Turkey, Africa and recently in some Asian countries. Commercial enzyme-linked immunosorbent assay (ELISA) kits were widely used by researchers in many epidemiological studies and SBV diagnosis. The aim of this study was to optimise indirect in-house ELISA that is based on different antigen preparations of cell-culture derived whole SBV particle. Antigen preparations were maintained with various methods: PEG precipitation, ultracentrifugation, dialysis, and antigen inactivation. Following antigen optimisation, steps of antigen coating, blocking, conjugate and stop solution were optimised and in-house ELISA was compared to commercial indirect SBV ELISA kit. The best result in ELISA antigen preparation for SBV was gained by 30% PEG purification method followed by formaldehyde inactivation. Although results of this study demonstrated that in-house ELISA for detection of SBV specific antibodies was equally sensitive and specific as commercial kit, purified SBV antigen based in-house ELISA development could increase S/P ratios.

Keywords: *Diagnosis, Dialysis, ELISA, PEG, Schmallenberg virus*

Schmallenberg Virüs Antikorlarının Belirlenmesi İçin İndirekt ELISA'nın Farklı Antijenler Karşılaştırılarak Optimizasyonu

Öz

Schmallenberg virüs (SBV) enfeksiyonu 2011 yılında keşfedilmiş ve enfeksiyon Türkiye dahil Avrupa kıtasında, Afrika'da ve bazı Asya ülkelerinde bildirilmiştir. Ticari ELISA kitleri çok sayıda epidemiyolojik çalışmada ve SBV tanısında sıklıkla kullanılmaktadır. Bu çalışmanın amacı, hücre kültüründen elde edilen tam SBV partikülünün farklı antijen hazırlama yöntemleri kullanılarak indirekt in-house ELISA optimizasyonudur. Antijen hazırlamak için; PEG presipitasyon, ultrasantrifüjleme, diyaliz ve antijen inaktivasyonu gibi farklı yöntemler kullanıldı. Antijen optimizasyonu sonrasında, antijen kaplama, bloklama, konjugat ve durdurma solüsyonları optimize edildi ve geliştirilen in-house ELISA ticari ELISA kiti ile kıyaslandı. ELISA antijen hazırlanmasında en iyi sonuçlar %30 PEG presipitasyon sonrasında formaldehit ile inaktivasyon sonucunda elde edildi. SBV özgül antikorların belirlenmesinde in-house ELISA ticari kit kadar duyarlı ve özgül olsa da saf SBV antijeni temelli in-house ELISA geliştirilmesinin S/P oranını yükseltebileceği düşünüldü.

Anahtar sözcükler: *Tanı, Diyaliz, ELISA, PEG, Schmallenberg virüs*

INTRODUCTION

Schmallenberg virus (SBV), first identified in 2011 in Germany, is classified in *Orthobunyavirus* genus of *Peribunyaviridae* family. SBV causes abortion, stillbirths, and congenital

malformation in ruminants ^[1,2]. Since 2011, SBV infection in wild and domestic ruminants was reported in many countries in Europe including Turkey ^[2-6]. SBV infection was found in some African and Asian countries recently ^[7-9]. Seroepidemiological data of SBV infection in Turkey



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revealed that SBV seropositivity in cattle, sheep, goats, and Anatolian water buffalo was 39.8%, 1.6%, 2.8%, and 1.5%, respectively [3]. SBV seropositivity in some European countries was estimated to be up to 98.5% in cattle, 89% in sheep, and 50.8% in goat [10]. SBV infection has been detected in both domestic and wild ruminants, including cattle, sheep, goat, buffalo, deer, and bison [3].

There are many assays developed for diagnosis of SBV infection to date. Reverse transcriptase PCR (RT-PCR), real-time RT-PCR assays, loop-mediated isothermal amplification (LAMP), and recombinase polymerase amplification (RPA) are developed for molecular diagnosis of SBV [11-13]. For detection of SBV specific antibodies, virus neutralisation, plaque reduction neutralisation, and enzyme-linked immunosorbent assay (ELISA) tests were used [14-17]. Indirect and competitive ELISA kits which are based on nucleoprotein (N) of SBV are commercially available for detection of SBV specific antibodies in serum, plasma and milk samples. The commercial kits are used by many researchers to determine SBV seropositivity for seroepidemiological surveys and diagnosis [3,18]. On the other hand, some researchers developed in-house ELISA to determine SBV specific antibody based on whole virus particle [16,17]. In this study it is aimed to optimise an indirect ELISA assay based on cell-culture derived whole viral particle for serological diagnosis of SBV infection by preparation and comparison of different SBV ELISA antigens.

MATERIAL and METHODS

Schmallenberg Virus

Schmallenberg virus isolate (strain F6; GenBank accessions: KC355457-KC355459) was kindly provided by Prof. Dr. Wim van der Poel (Wageningen Bioveterinary Research, Netherlands). SBV was propagated on Vero cells which were grown using Dulbecco modified Eagle medium (DMEM) with 10% foetal bovine serum (FBS) and incubated at 37°C with 5% CO₂. The viral titer was determined by plaque titration assay as described previously [19].

Serum Samples

Serum samples taken from 300 cattle in previous studies were tested with both commercial indirect ELISA kit (IDEXX, Westbrook, Maine, USA) and competitive ELISA kit (IDVet, Grabels, France) in order to determine SBV-seropositive and SBV-seronegative samples. Both commercial kits were used as following the instructions of the manufacturers. The positive and negative sera according to results of these commercial kits were accepted as SBV-seropositive and SBV-seronegative samples. In-house ELISA results were compared to commercial indirect ELISA results.

Preparation of ELISA Antigen

Vero cells were infected with SBV and cells were frozen after 80% or over of cells showed cytopathic effect. Freeze-thaw

process was maintained for 2-3 times. Different methods which have implemented and/or not been implemented before such as, polyethylene glycol (PEG) precipitation, ultracentrifugation, dialysis, and inactivation for antigen preparation were carried out.

Precipitation with 50% PEG: 50% PEG 8000 (w/v) (Sigma, Missouri, USA) and 23% NaCl (w/v) (Merck, Darmstadt, Germany) were dissolved in distilled water. Supernatant of SBV infected cells was collected and centrifuged at 3000 rpm for 30 min at 4°C. The supernatant was mixed with 50% PEG and 23% NaCl solution was added to the mixture. Virus-PEG mixture was stirred at 100 rpm for 16 h at 4°C. The mixture was centrifuged at 12000 rpm for 30 min at 4°C. Pellet was suspended in 1× TNE buffer (pH 7.2) and centrifuged at 5500 rpm for 20 min at 4°C. After centrifugation supernatant was collected and centrifuged at 24000 rpm for 2 h at 4°C. Supernatant was discarded and the pellet was suspended in 1× TNE buffer and stored at -80°C until used [20].

Precipitation with 30% PEG: Viral supernatant was mixed at a ratio of 2:1 with 30% PEG 8000 (w/v) which was prepared in 0.4 M NaCl [21]. The mixture was incubated at 4°C overnight. The virus was recovered by centrifugation at 3200×g for 30 min at 4°C. Supernatant was discarded and the virus was suspended in PBS and stored at -80°C until used.

Ultracentrifugation: Supernatant of SBV infected cells was collected and centrifuged at 2000 rpm for 10 min at 4°C. After centrifugation, supernatant was collected and filtered with using 0.22 µm pore filter membranes. The filtrate was ultracentrifuged (in Department of Virology, Faculty of Veterinary Medicine, Firat University, Elazig, Turkey) using SW-28 rotor (Beckman Coulter, Brea, California, USA) at 25.000 rpm for 2 h at 4°C. DMEM containing 1% BSA was pour onto the viral pellet, incubated for 2 h at 4°C, and re-suspended.

Commercial PEG precipitation kit: SBV antigen was purified with PEG virus precipitation kit (Biovision, California, USA) according to manufacturer's instruction. Briefly, cells infected with SBV were centrifuged at 3200 ×g for 15 min at 4°C. Supernatant was collected, mixed with 5× PEG solution and incubated overnight at 4°C. The mixture was centrifuged the following day at 3200×g for 30 min at 4°C. The viral pellet was suspended in virus suspension buffer and stored at -80°C until used.

Dialysis: Dialysis was carried out with two different systems: 12-14 kDa cut-off dialysis tubes (Biovision, California, USA) and Spectra/Por2 dialysis membrane 12-14 kDa MWCO (Spectrum, Waltham, Massachusetts, USA). The dialysis tubes were soaked with distilled water and the tubes were dialyzed against water overnight at 4°C and against PBS for 4 h at 4°C [17]. After dialysis, virus collected and stored at -80°C until used. The dialysis membrane was initially

soaked in distilled water for 30 min. The supernatant was dialyzed against type I MilliQ water during day and overnight at 4°C and water was changed at least 4 times. Final dialysis was carried out against PBS overnight at 4°C [21]. The virus was collected and stored at -80°C until used.

Antigen inactivation: To inactivate SBV, the antigens were treated by Triton X-100 with final concentration of 1% [17] or formaldehyde with final concentration of 2% [22] and incubated in room temperature for 1 h.

SDS-PAGE and Western Blotting

SDS-PAGE and western blotting was carried out to confirm SBV isolate and antigen preparation method and to determine SBV-seropositive serum samples were reacting against viral proteins. For separation of protein suspensions, protein electrophoresis was carried out in 10% SDS-PAGE gel. The proteins were transferred onto a PVDF membrane (Thermo Scientific, Waltham, Massachusetts, USA) and the membrane was blocked overnight at 4°C with 5% skimmed milk powder in phosphate buffered saline with 0.05% Tween-20 (PBST). SBV-seropositive and -seronegative cattle sera were used as primary antibody and the membrane was incubated in sera diluted 1:100 in 0.01% PBST at room temperature for 2 h. Washing step was carried out with 0.1% PBST for three times. Rabbit anti-bovine IgG HRP secondary antibody (Life Technologies, Carlsbad, California, USA) diluted 1:1000 in 0.01% PBST was added onto the membrane and incubated at room temperature for 1 h. Following washing with 0.1% PBST, TMB substrate (Amresco, Solon, Ohio, USA) was poured onto the membrane, incubated for 5 min and evaluated with pre-stained protein marker (Thermo Scientific, Waltham, Massachusetts, USA).

In House Indirect ELISA Optimisation

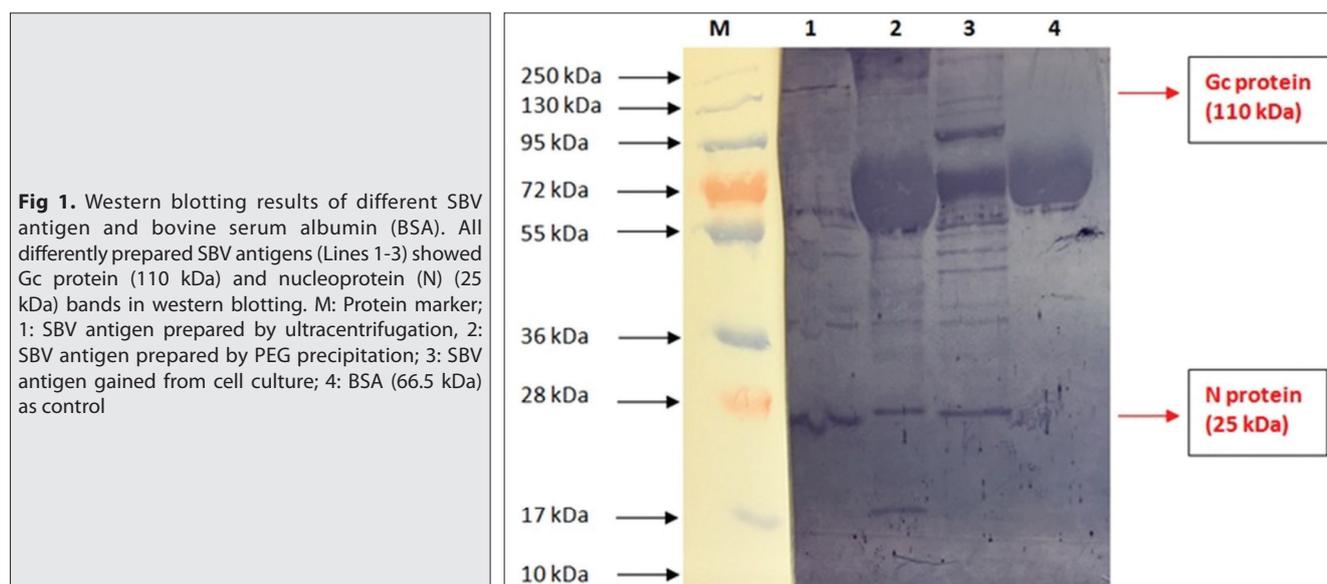
Concentrations of differently prepared antigens were measured by Bradford protein assay kit (Thermo Fisher

Scientific, Waltham, Massachusetts, USA). In-house indirect ELISA was optimised by performing checkerboard titrations of antigen and conjugate using positive and negative serum samples which were defined by commercial indirect and competitive ELISA kits. Further optimisations of SBV antigen, coating buffer, blocking, diluents, and stop solution were implemented for the in-house indirect ELISA. SBV antigen was diluted in both 0.05 M carbonate/bicarbonate buffer and PBS. Fifty µL of antigen solution at 10 µg/mL concentration was coated onto polystyrene ELISA plates by incubating at 4°C overnight. Undiluted sera, 1/2, 1/10 and 1/100 dilutions of sera are tested for sample step. The washing steps were carried out with 0.05% PBST. Two different blocking agents, 10% skimmed milk powder and 1:10 diluted foetal bovine serum (FBS), were tried for blocking step. The conjugate (Life Technologies, Carlsbad, California, USA) was diluted in different diluents, such as 5% skimmed milk powder (in 0.05% PBST) and 3% FBS (in 0.05% PBST). TMB substrate was used and the reaction was stopped by using 2 M sulphuric acid or 1% sodium dodecyl sulphate (SDS). Results were read at 450 nm using a spectrophotometer.

RESULTS

Three hundred cattle sera were tested by both commercial indirect and competitive ELISA kits and 22 out of 300 sera were positive for SBV specific antibody in both commercial kits. Positive and negative sera were used for development of in-house indirect ELISA. Differently prepared ELISA antigens were tested by SDS-PAGE and western blotting and SBV nucleoprotein (25 kDa) and Gc protein (110 kDa) were detected with SBV seropositive serum (Fig. 1), whereas SBV seronegative serum cannot detect any SBV proteins by western blotting analysis (data not shown).

After checkerboard titrations of antigen and conjugate, optimisation steps of SBV antigen, coating buffer, blocking



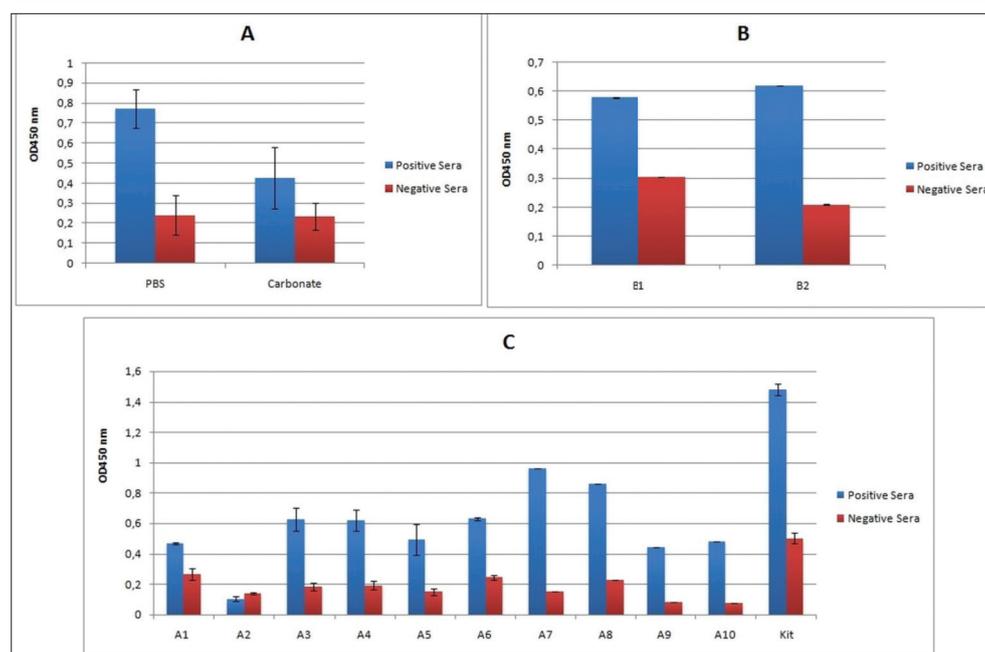


Fig 2. Comparison of optic density (OD) results of in-house ELISA for detection of SBV specific antibodies in cattle sera. **A:** Carbonate buffer and PBS were compared for antigen coating step, **B:** Blocking step was interpreted by 10% skimmed milk powder and 1:10 diluted FBS. B1: blocking with 10% skimmed milk powder, B2: blocking with 1:10 diluted FBS, **C:** Differently prepared ELISA antigens were compared to each other and indirect ELISA kit. SBV ELISA antigens were differently prepared; A1: 50% PEG, A2: ultra-centrifugation, A3: PEG kit, A4:30% PEG, A5: 30% PEG and dialysis, A6: dialysis and PEG, A7: 30% PEG and formaldehyde, A8: PEG kit and formaldehyde, A9: 30% PEG and triton X-100, A10: PEG kit and triton X-100. Kit: Indirect ELISA kit for SBV

Table 1. The S/P% results of in-house indirect ELISA were calculated with formulation of indirect ELISA kit

| Antigens | Positive Sera, S/P% (mean±SD) | Negative Sera, S/P% (mean±SD) |
|---------------------------------------|-------------------------------|-------------------------------|
| 30% PEG | 53.0±2.181 | 9.0±0.371 |
| 30% PEG and Triton X-100 inactivation | 21.3±0.876 | -12.8±0.525 |
| 30% PEG and formaldehyde inactivation | 69.9±2.877 | -6.3±0.259 |
| PEG kit | 40.6±1.668 | 4.6±0.189 |
| PEG kit and triton X-100 inactivation | 24.8±1.019 | -13.3±0.548 |
| PEG kit and formaldehyde inactivation | 60.4±2.483 | 1.0±0.042 |

The indirect ELISA kit results were calculated and interpreted as positive if S/P% ≥40%, as doubtful if 30% ≤S/P%<40%, and as negative if S/P%<30%

agent, and stop solution were implemented. SBV antigen was diluted at concentration of 10 µg/mL and coated successfully in PBS, not in 0.05 M carbonate/bicarbonate buffer. Antigen coating with PBS results in OD of 0.773±0.094 for positive sera and 0.241±0.098 for negative sera, whereas carbonate/bicarbonate buffer coating results in 0.4255±0.152 for positive sera and 0.234±0.067 for negative sera (Fig. 2-A). Because coating with PBS resulted in a higher OD value for positive sera, PBS was preferred for the antigen coating step.

To optimise blocking step of in-house ELISA, skimmed milk powder and FBS were tested. The positive sera OD was 0.579±0.001 and negative sera OD was 0.3045±0.0015 in blocking with 10% skimmed milk powder. The OD values of blocking with 1:10 diluted FBS were 0.6205±0.0005 for positive sera and 0.209±0.001 for negative sera (Fig. 2-B). For blocking step, 1:10 diluted FBS has the best OD results.

For sample step, undiluted sera, 1/2 and 1/10 dilutions of sera gave same O.D. results, whereas 1/100 dilution O.D. was undetectable. Therefore, optimisation of sample step was implemented with 1/10 dilutions of sera.

Skimmed milk powder and FBS were used as diluents of the conjugate to optimise conjugate step of in-house ELISA. The optimisation of conjugate step was maintained in commercial indirect ELISA kit (IDEXX, Westbrook, Maine, USA) with following the instructions of the manufacturer, except for conjugate step which was carried out with three different conjugates. The first one is the conjugate of commercial kit (IDEXX), the second one is the conjugate (Life Technologies) diluted 1:2000 in 5% skimmed milk powder, and the third one is the conjugate (Life Technologies) diluted 1:2000 in 3% FBS (in 0.05% PBST). When the full procedure of commercial ELISA kit was followed, the OD of positive sera and negative sera were 1.5905±0.0355 and 0.2545±0.0125, respectively. Once the conjugate (Life Technologies) was diluted 1:2000 in 5% skimmed milk powder (in 0.05% PBST), the OD values were 0.5105±0.0125 for positive sera and 0.2455±0.0035 for negative sera. The most optimised results gained by 1:2000 dilution of the conjugate (Life Technologies) in 3% FBS (in 0.05% PBST), with OD values of 2.2125±0.0235 and 0.3155±0.0205 for positive and negative sera, respectively. ELISA reactions were stopped by adding 2M sulphuric acid, but not with 1% SDS.

To find the best antigen preparation system, 50% PEG and 30% PEG precipitation, ultracentrifugation, commercial PEG precipitation kit, dialysis, and antigen inactivation (Triton X-100 or formaldehyde) methods were implemented and compared to each other. Comparison of OD values of the different antigens indicated that the ELISA antigen gained by 30% PEG precipitation with formaldehyde inactivation method has the highest efficiency as having 0.961 and 0.149 mean OD values for positive and negative sera, respectively (Fig. 2-C). However, the result of commercial indirect ELISA kit has higher OD for positive sera (mean OD of 1.481). The S/P% values of different antigens-based in-house ELISA were given in Table 1.

Following optimisation of steps of in-house ELISA, results were compared with commercial indirect ELISA kit (IDEXX, Westbrook, Maine, USA) (Fig. 2-C). All positive and negative sera which were already determined with two commercial kits were positive and negative, respectively, with in-house ELISA; however, the OD values were higher in commercial indirect ELISA kit in comparison to OD values of in-house ELISA (Fig. 2-C). The sensitivity and specificity of in-house ELISA were determined as 100% when compared to commercial ELISA kits.

DISCUSSION

ELISA is one of the most commonly used serological techniques and the use of ELISA in serosurveys for viral diseases provides convenience to the scientists to assess the epidemiology, and rate of spread of the diseases. Since the first discovery of SBV infection in 2011, ELISA is widely used for SBV diagnosis and seroepidemiology. Commercial ELISA kits are available and in-house ELISAs are developed by some researchers. In present study, it is aimed to optimise indirect in-house ELISA for SBV antibody detection with assessing the results of different antigens and diluents which have not tested for SBV ELISA to date.

In the present study, different ELISA antigen preparation methods were implemented: precipitation with 50% PEG, precipitation with 30% PEG, commercial PEG precipitation kit, dialysis, and antigen inactivation with Triton X-100 or formaldehyde. Among these methods, the best ELISA antigen was prepared by 30% PEG purification following inactivation with formaldehyde in the present study. A previously used method for inactivation of SBV [17] with Triton X-100 has been resulted in low OD value when compared to formaldehyde inactivation method (Fig. 2-C). Besides the methods used in the present study, sucrose gradient, caesium chloride density gradient, ultrafiltration could be applied for virus purification. Sucrose gradient method is found to be better for foot-and-mouth disease virus [23]. Some combination of purification methods could increase viral yield, such as sucrose gradient following dialysis and ultrafiltration method found to have the

best yield for norovirus [24]. In the present study some combination of methods were tested and the antigen gained by 30% PEG purification method in combination with formaldehyde inactivation increased OD results (Table 1). ELISA for detection of virus specific antibodies can be based on either whole virus [16,17] or recombinant protein of the virus [25,26]. In the present study, only whole SBV was implemented in indirect ELISA. Using the whole virus as antigen in ELISA may bring the risk of cross-reaction with other Orthobunyaviruses and this can be overcome by using pure SBV antigen.

ELISA antigen coating could be maintained by PBS and carbonate/bicarbonate buffer. It is known that using a coating buffer with pH of 1-2 units higher than the isoelectric point of the antigen could increase binding of the ELISA antigen [27]. Because isoelectric point of SBV still remains unknown, both PBS and carbonate/bicarbonate buffer were tested for in-house ELISA in the present study. Antigen coating step was successfully maintained in PBS in the present study, although other researchers used carbonate/bicarbonate buffer for coating of SBV antigen [16,17].

Foetal bovine serum, skimmed milk powder, normal goat serum, normal chicken serum, bovine serum albumin (BSA), and gelatine could be used as blocking agent in home-made ELISAs [27]. In this study blocking with 1:10 diluted FBS had the best results in comparison to 10% skimmed milk powder. Conjugate dilution buffer may differ among studies, e.g. PBS with 0.05% Tween 20, PBS with 0.05% Tween 80, 5% FBS, 1% BSA could be used for conjugate dilution [16,17,25,26]. Several diluents and concentrations were tried out for conjugate optimisation in the present study. Conjugate was diluted in 5% skimmed milk (in 0.05% PBST) and 3% FBS (in 0.05% PBST) with different concentrations. The most optimised result was gained by 1:2000 dilution of conjugate in 3% FBS (in 0.05% PBST).

In conclusion, an in-house ELISA for detection of SBV-specific antibodies was optimised with the antigen gained by 30% PEG purification following inactivation with formaldehyde. In further studies, different methods such as sucrose gradient, caesium chloride density gradient, and ultrafiltration can be tested for SBV ELISA antigen.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

STATEMENTS AUTHORS CONTRIBUTIONS

AKA, EA designed the experiments, made serum samples, the preparation of ELISA antigen stages, and SDS-PAGE-western blotting, optimization works and wrote the manuscript. AKA, made a substantial contribution to interpretation of data. All authors discussed the results and contributed to the final manuscript.

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