Establishment of an Indirect Enzyme-linked Immunosorbent Assay for Detection of the NS4 Protein of Bluetongue Virus

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Abstract

An indirect enzyme-linked immunosorbent assay (iELISA) was established to detect the serological prevalence of bluetongue virus (BTV) infection in ruminant populations. A recombinant NS4 (rNS4) protein was used as the encapsulated antigen. Optimization of the iELISA included the encapsulated antigen, serum dilution, blocking solution, and working concentration of a horseradish peroxidase (HRP)-labeled secondary antibody (Ab) by the square-matrix titration test. The results showed that the optimal reaction conditions for the iELISA were: volume of coating buffer, 2.5 µg/mL; serum dilution, 1:200; blocking solution, 0.25% polyvinyl alcohol + 5% skim milk powder; working concentration of the HRP-labeled secondary Ab, 1:4000; and critical values, sample-to-positive ratios 0.26 and 0.38 for determining positivity and negativity, respectively. The detection sensitivity of the iELISA was up to 1:4000, the intra- and inter-batch reproducibility coefficients of variation were less than 10%, and the positive and negative concordance rates of 56 bovine serum samples were both 100%, confirming excellent sensitivity and reproducibility. The proposed iELISA should prove useful for the diagnosis of BTV infection and future epidemiological investigations.

Introduction

Bluetongue (BT) is an infectious disease of ruminants caused by the bluetongue virus (BTV), which is transmitted by biting midges in the genus *Culicoides*. The clinical manifestations of BT disease range from asymptomatic infection to fatal hemorrhagic fever. BTV infection of sheep is characterized by severe clinical symptoms, while infected goats, deer, and cattle are usually asymptomatic. BTV replicates and persists in ruminants, thus cryptically infected animals are the main source of infection and spread of BT disease [1,2]. Although there is no curative treatment at present, an inactivated vaccine is widely used internationally to inoculate susceptible animals to prevent and control BT disease. An inactivated vaccine against BTV-1 developed by the Yunnan Kunming Academy of Animal Husbandry and Veterinary Science (Kunming, China)

invokes a good immunological response, but it remains difficult to distinguish infected animals from those immunized with a vaccine [3-6]. In addition to the development of a new vaccine, it is important to establish a detection method to assess immunization status and identify infected animals to prevent and control the spread of BTV.

BTV is a non-enveloped virus composed of two concentric protein shells that encapsulate 10 double-stranded RNA genome segments that encode seven structural proteins (VP1–VP7) and five nonstructural proteins (NS1–NS5). The NS4 gene encodes a protein composed of 77–79 highly conserved amino acids [7-9]. BTV VP7, which exists as a trimer within the viral nucleocapsid, is a BTV group-specific antigen. Most serological assays for detection of BTV are based on the detection of antibodies (Abs) against VP7. Current assays for

detection of BTV include competitive indirect enzyme-linked immunosorbent assays (iELISAs) based on VP7 [14], double Ab sandwich ELISAs [15], c-ELISAs [16,17], iELISAs [18], a blocking ELISA based on recombinant core-like particles [19], an ELISA for identification of wild-type BTV [20], and an antigen-based c-ELISA [21]. However, ELISAs based on the structural proteins of BTV cannot distinguish between animals infected with a wild-type virus and those immunized with an inactivated vaccine because immunization with such vaccines does not evoke the production of antibodies against non-structural proteins [22]. Therefore, an assay based on a nonstructural protein is needed.

NS4 is a key determinant of BTV virulence and was found to inhibit intracellular production of type I interferons (IFN-I) and the expression of multiple interferon genes [9-12]. In view of the important role of NS4 in antagonizing natural immunity of the host, an iELISA was developed for detection of a purified pET-32a-NS4 fusion protein as an encapsulated antigen. In addition, optimization of the iELISA included the amount of the encapsulation antigen, serum dilution, blocking solution concentration, working concentration of a horseradish peroxidase (HRP)-labeled secondary Ab, and reaction threshold for early detection of BTV.

Materials and Methods

Serum samples and primary reagents

BTV-negative and -positive control serum samples in addition to 30 serum samples from BTV-infected cattle and 26 from noninfected cattle were provided by Chongqing Yongchuan Center for Animal Disease Prevention and Control (Chongqing, China) and Chongqing Nabii Veterinary Diagnostic and Technical Services Co. Ltd. (Chongqing, China). Isopropyl ß-D-1-thiogalactopyranoside was purchased from TaKaRa Biotechnology (Dalian, China). A dialysis bag was obtained from EMD Millipore Corporation (Billerica, MA, USA). Costar® 96-well microtiter plates were acquired from Corning Incorporated (Corning, NY, USA). HRP-labeled rabbit anti-bovine immunoglobulin G (IgG) was purchased from United States Biological (Salem, MA, USA). Nickel ion–iminodiacetic acid affinity chromatography resin and a protein purification kit

were obtained from Novagen (Madison, WI, USA). The Spectra™ Multicolor Broad Range Protein Ladder was purchased from Thermo Fisher Scientific (Waltham, MA, USA). A BTV-specific antigen-capture ELISA (c-ELISA) kit was obtained from Yunnan Animal Science and Veterinary Institute (Kunming, China). All other reagents were of analytical grade.

Instrumentation

A multifunctional microplate reader was purchased from Tecan Trading (Shanghai, China). All other instruments and equipment were provided by Chongqing Veterinary Science Engineering Center (Chongqing, China).

Methodology

Optimization of the encapsulated antigen and serum dilution: The purified recombinant NS4 (rNS4) protein, as the encapsulated antigen, was diluted with carbonate buffer to concentrations of 5, 2.5, 1.25, 0.625, 0.3125, and 0.15625 µg/mL. The BTV-positive and -negative serum samples were diluted to 1:50, 1:100, 1:200, and 1:400, and the positive/negative (P/N) values were compared. The optical density at 450 nm (OD_{450}) of the BTV-positive serum samples approached 1.0. The maximum P/N value was calculated to determine the optimal amount of the encapsulated antigen and serum dilution.

Optimal blocking solution: After encapsulation, the wells were washed once with carbonate buffer, followed by the addition of 300 µL of blocking solution per well, as described in **Table 1**. Optimization of the other parameters was conducted in accordance with the conditions described in section 2.3.1. The optimal blocking conditions for the wells were based on the largest P/N value.

Screening of the working concentration of the HRP-labeled Ab: The HRP-labeled rabbit anti-bovine IgG Ab was diluted to 1:1000, 1:2000, 1:4000, and 1:8000 to determine the optimal working concentration.

Determination of reaction critical values: In accordance with the optimal reaction conditions described above, 10 BTV-negative serum samples were screened in triplicate. The

Table 1. Blocking buffer and blocking conditions.

Blocking buffer	Blocking conditions
0.25% PVA solution	37°C for 2 h
5% skimmed milk powder	37°C for 2 h
1% bovine serum albumin	37°C for 2 h
2% seaweed sugar	37°C for 2 h
0.25% PVA solution + 5% skimmed milk powder	Blocking with 0.25% PVA solution for 2 h at 37°C, followed by 5% skimmed milk powder at 37°C for 30 min

absorbance (OD_{450}) of the serum samples was measured with a microplate reader and the mean ($\bar{X}$) ± standard deviation (SD) was calculated for comparisons. Also, the optimal conditions to determine negative and positive results were elucidated.

Sensitivity: The BTV-positive serum samples were diluted to 1:50, 1:100, 1:200, and 1:400 to test the reaction conditions. The maximum dilution of the positive sera was used to assess the sensitivity of the iELISA.

Reproducibility: In accordance with the optimal reaction conditions, 20 serum samples were tested using the same batch of coated plates and five different batches of coated microtiter plates, including 10 each for the BTV-positive and -negative serum samples. The iELISA was repeated five times and the $\bar{X}$ ± SD of the absorbance values was calculated to assess the reproducibility of the iELISA.

Clinical sample compliance: The iELISA established in section 2.3 was further tested for detection of the rNS4 protein in 56 bovine serum samples (30 BTV-positive and 26 BTV-negative) and the results were compared to those of a commercial BTV c-ELISA Ab detection kit.

Results

Optimization of the amount of the coating antigen and serum dilution

The gradients of the rNS4 protein and diluted serum are shown in **Table 2**. The OD_{450} values of the BTV-positive and -negative serum samples were measured and the corresponding P/N values were calculated. The highest P/N value was 19.60. The optimal amount of the coating antigen was 2.5 µg/mL and the optimal serum dilution was 1:200 (**Table 3**).

Optimal composition of the blocking solution

The combination of 0.25% polyvinyl alcohol (PVA) and 5% skim milk powder was selected as the optimal composition of the blocking solution. The P/N value of the BTV-positive and -negative serum samples reached 15.87.

Determination of the optimal working concentration of the HRP-labeled Ab

The HRP-labeled Ab was diluted to 1:1000, 1:2000, 1:4000,

Table 2. Optimization of the coating rNS4 protein and serum dilution by the square-matrix titration test.

Amount of antigen (µg/mL)	BTV-positive serum				BTV-negative serum				
	1:50	1:100	1:200	1:400	1:50	1:100	1:200	1:400	Blank control
5.0	1.3018[a]	1.0275	0.9295	0.6912	0.1289	0.0858	0.0695	0.0407	0.0302
2.5	1.1785	0.9732	0.8939	0.5675	0.0797	0.0679	0.0456	0.0310	0.0309
1.25	1.0808	0.8998	0.7576	0.4729	0.0603	0.0751	0.0449	0.0302	0.0428
0.625	0.8375	0.6943	0.5967	0.2817	0.0547	0.0532	0.0429	0.0300	0.0304
0.3125	0.5524	0.4706	0.2756	0.1719	0.0505	0.0455	0.0416	0.0318	0.0211
0.1563	0.3578	0.2896	0.1661	0.1009	0.0409	0.0412	0.0400	0.0305	0.0218

[a] Absorbance values are shown in the table.

Table 3. P/N values of the rNS4 protein.

Amount of rNS4 protein (µg/mL)	P/N value			
	1:50	1:100	1:200	1:400
5.0	10.09	11.97	13.37	16.99
2.5	14.79	14.33	19.60	18.31
1.25	17.92	11.98	16.87	15.65
0.625	15.31	13.05	13.90	9.39
0.3125	10.93	10.34	6.63	5.40
0.1563	8.74	7.07	4.15	3.30

and 1:8000. Although there was no significant difference in the P/N values among the first three dilutions, at 1:8000 the P/N value decreased significantly. Therefore, 1:4000 was determined as the optimal working concentration of the HRP-labeled Ab.

Determination of the reaction critical values

The iELISA was tested using 10 BTV-negative serum samples, with $\bar{X} \pm 3$ SD as the upper threshold, $\bar{X} \pm 2$ SD as the lower threshold, and values in between considered as suspicious. Combining the above conditions, the serum sample is considered positive at a sample-to-positive (S/P) ratio of >0.38, negative at <0.26, and suspicious from 0.26 to ≤ 0.38.

Sensitivity

The iELISA established in this study was used to detect the rNS4 protein in BTV-positive serum samples at different dilutions. The results showed that the values determined from dilutions of 1:50, 1:100, 1:200, 1:400, 1:800, and 1:1600 were still higher than the threshold values, indicating that the iELISA is sensitive.

Repeatability

In total, 20 serum samples (10 BTV-positive and 10 BTV-negative) were tested using five different batches of coated microtiter plates. The coefficients of variation were <10%, indicating good repeatability of the established iELISA (see **S1 Table** and **S2 Table** for specific data).

Consistency with clinical serum samples

The consistency of the proposed iELISA for detection of the rNS4 protein was tested using 56 clinical bovine serum samples (30 BTV-positive and 26 BTV-negative). All 30 BTV-positive serum specimens yielded positive results for the NS4 Abs, corresponding to a positive concordance rate of 100%, while all 26 BTV-negative serum samples were negative for the rNS4 Abs and none were considered suspicious, corresponding to a negative concordance rate of 100%.

Discussion

BTV is classified as notifiable animal infectious diseases by the World Organization for Animal Health. In China, BTV is predominantly a recessive infection. However, when different field strains of BTV are present in the same area at the same time, BTV is prone to genetic recombination, which leads to increased genetic diversity. Under these circumstances, it is impossible to predict the risk of infection to susceptible animals [13,14]. Therefore, it is important to develop simple, rapid, and sensitive diagnostic methods for large-scale detection. The proposed iELISA is a convenient, rapid, sensitive, and specific assay that is simple to standardize.

The NS4 protein of BTV-1 can coordinate with the NS3 protein to directly interact with and impair phosphorylation of signal transducer and activator of transcription 1 (STAT1) in a dose-dependent manner, thereby interfering with IFN-I signaling in the JAK-STAT pathway, which facilitates the ability of BTV to evade the natural immune response of the host cell [23]. NS4 and NS3 are both composed of conserved amino acid sequences and are detected in BTV-infected cells. Huang et al. [20] established an iELISA for detection of BTV based on a rNS3 protein with a sensitivity of 1:1280, intra- and inter-batch coefficients of variation of less than 10%, and 100% compliance with commercial assay kits for detection of BTV-positive samples. The good specificity, sensitivity, and reproducibility of this assay allows for detection of vaccine-acquired immunity against BTV and infection of the wild-type virus. Given that NS4 also plays an important role in viral infection of host cells, this iELISA was constructed by encapsulating the BTV NS4 protein. The optimized reaction conditions were: serum dilution, 1:200; blocking solution, 0.25% PVA + 5% skim milk powder; working concentration of the HRP-linked secondary Ab, 1:4000; and reaction critical values, 0.26 and 0.38. The results of 56 clinical samples showed that the compliance rate of BTV-positive serum samples was 100%, indicating excellent sensitivity and reproducibility. Our group plans further investigations to determine whether the proposed iELISA can differentiate between serum samples with Abs produced by vaccine-acquired immunity and those collected from animals infected the wild-type virus to lay a foundation for the prevention, diagnosis, and control of BTV infection.

Conflict of Interest

The authors declare that there are no conflicts of interest that could have influenced the outcome of this study.

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