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Improved Efficiency of Bluetongue Viral Antigen Isolation for Successful Immunization

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ABSTRACT

Bluetongue (BT) is a viral disease transmitted by Culicoides spp. The clinical presentation of BT varies widely among susceptible sheep, and in most cases results in severe illness and death in the infected animals. The mortality among susceptible sheep ranges from 2-30% but can occasionally be as high as 70%. Therefore, we investigated a new method to increase the purified BTV-antigen. BTV viral suspensions were purified using Freon-113 and ultracentrifugation through 40% sucrose. We obtained 94.5-95.8% purification of the BT-16 viral antigen. Sheep and cows were immunized with the isolated BTV antigen obtained from this method to confirm antibody specificity to BTV. The antibody activity measured by enzyme-linked immunosorbent assay (ELISA) from goat serum was 7.0log^2 to 13.0log^2 (on average 10.8±2.28log^2) relative to that of sheep ($P<0.05$ to $P<0.0001$). We show here that this method can successfully purify BTV-16 antigen and could be used for large-scale production and other BTV serotypes.

Key words: Bluetongue Virus; Freon-113; Serum; Viral Purification; Agar Gel Immunodiffusion; Ruminants.

INTRODUCTION

Bluetongue virus (BTV) is a member of the family *Reoviridae* and genus *Orbivirus* and is the etiological agent responsible for bluetongue disease (BT). Epizootiological studies indicate BT is one of the most economically harmful infectious diseases in ruminants, particularly sheep (Sbizera et al. 2019). Currently, 27 BTV serotypes have been identified, with limited serological cross-neutralization and cross-protection similarities (Ries et al. 2020). Sheep, cattle, deer, camels, buffaloes, goats, and other wild ruminants are susceptible to BTV infection in their natural habitat (Zhumanov et al. 2015; Bulegenova et al. 2019). In susceptible animals, the disease is characterized by fever and hemorrhagic mucus membranes, which develop into inflammatory necrotic lesions in the mouth, tongue, gastrointestinal tract, corolla epithelium, the base of hooves, and swelling of the intermaxillary space and chest (Matthews et al. 2022; Selim et al. 2022).

A positive BT diagnosis is established based on clinical and pathological anatomical examinations (Bianchi et al. 2017; Kang et al. 2022; Sanders et al. 2022). Viral detection is determined by the presence of either viral mRNA determined by PCR or virus-specific antibodies from whole blood, organ, or tissues samples from suspected, infected animals (Maan et al. 2016). Serological diagnosis of BT is essential when understanding the disease mechanism and helps to identify asymptomatic carriers of BT. Serological tests such as a serum/virus neutralization test (SNT or VNT), complement fixation test, agar gel immunodiffusion (AGID), method of fluorescent antibodies, and an enzyme-linked immunosorbent assay (ELISA) are useful for retrospective diagnoses (Afshar 1994; Rojas et al. 2019). Importantly, the development of these methods requires the availability of purified viral antigen. It has been previously shown that the following methods are used for preconcentration of BTV from cell cultures, including low-speed and differential centrifugation,

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precipitation with zinc acetate, adsorption-elution on cellulose anion and cation exchangers and inorganic sorbents (iron oxide powder, calcium phosphate), and precipitation with polyethylene glycol (PEG) (Verwoerd 1970; Titov 1971; Sung and Roy 2021). The existing modern methods of purification and concentration are very expensive, and the traditional methods give false positive results due to the large amount of cellular and serum proteins. Therefore, in this work, we concentrated and purified the BTV-16 antigen from tissue culture supernatants and immunized sheep and goats with the purified BTV antigen to induce an antigen-specific antibody response. Although this technology is not fundamentally different from currently available methods, we combined this with purification methods and introduced specific modifications to produce specific BTV antiserum.

MATERIALS AND METHODS

The current study was carried out in accordance with the scientific and ethical regulations recommended by the European Parliament Directive (2010/63/EU). All study procedures were approved by the Ethical committee of Scientific Research Institute for Biological Safety Problems.

The BT virus was initially isolated from embryonated chicken eggs and passaged once in Vero cells. The BT virus, strain 16 (BTV-16) was used for antigen preparation in this study and was originally isolated in 2007 from the spleen of a sheep that died due to a BTV (Khurosan, Tajikstan). We obtained this strain from the 'Collection of Microorganisms' laboratory at the Research Institute for Biological Safety Problems (RIBSP, Gvardeiskiy, Kazakhstan). BTV-16 was grown on Vero cells to a titer of 6.75 log10 to reach a tissue culture infective dose (TCID)/mL.

BTV-16 virus was grown on Vero cells with DMEM medium (Thermo Fisher Scientific). The medium was supplemented with 10% fetal bovine serum (FBS) and antibiotics (Penicillin/Streptomycin). Cells were allowed to become 90% confluent before being infected with BTV. Prior to infection, one bottle of cells from each group was trypsinized, and the number of cells counted. BTV was diluted in complete DMEM and used to infect cells at a multiplicity of infection of 0.01 to reach a TCID. Roller cultivation was carried out in 3.0L of circular vessels on the INCUDRIVE D-I apparatus (Schuett Biotec GmbH, Germany) at a rotation speed of 12rpm. BTV infection rates were monitored daily and incubated until 90-100% cytopathic effect (CPE) developed. Once CPE was determined, cells were detached using a 1:1 solution of trypsin-EDTA (Thermo Fisher Scientific). PEG-6000 (AppliChem GmbH, Germany) was added to the detached cells to a final concentration of 5.0% (w/vol) and dissolved by mixing at 500rpm, and the entire solution was incubated overnight at 4ºC. After incubation, the precipitate was separated by centrifugation at 4.000rpm for 40min at 4°C. The precipitate was resuspended in 100mL of 0.1M phosphate buffer solution (pH 7.2-7.4, Sigma-Aldrich, China) and subjected to 3 heat cycles (from RT, 23±3º-70ºC). The clarified suspension was obtained through centrifugation at 4.000rpm for 30mins at 4°C. The supernatant was transferred to a new tube for BTV antigen purification with Freon-113 (1,1,2-Trichlorotrifluoroethane, Fluka AG, Switzerland). Freon-113 was added in a 1:2 ratio of concentrated viral suspension. The mixture was vigorously shaken for 10min and centrifuged at 3.000rpm for 15min at 4°C. After centrifugation, the aqueous top phase containing the virus was harvested. To precipitate the virus, equal volumes of 0.002M Tris buffer pH 7.5 were added, and the mixture was shaken for 5min at RT, and centrifuged under the same conditions. BTV is prone to form aggregates, especially under ultracentrifugation, making it difficult to separate from the sediment. To improve the separation of the centrifuged virus, we pelleted the viral solution through a layer of 40% w/vol sucrose solution (Sigma-Aldrich, China). To further improve viral precipitation, NaCl (Araltuz, Kazakhstan) was added to a final concentration of 0.1M.

After ultracentrifugation, the virus-containing region of the supernatant was harvested and diluted 5times in 0.002M Tris buffer, pH 7.5. The sucrose cushion was then removed by precipitating the virus at 25.000rpm for 60 minutes at 4°C. The obtained viral pellet was resuspended in the same buffer in volume 0.5mL. Ten-fold limited dilution of BTV was done in complete DMEM, and 50µl of each dilution was plated on a 96-well cell culture plate. 1 x 10^5 Vero cells were added per well followed by incubation at 37°C with 5% CO_2 until CPE was observed. The viral titers were expressed in log10, and the TCID was calculated using the method described by Reed and Muench (1938).

To obtain a specific BTV antiserum, we used sheep (n=8) and goats (n=8) of a local breed aged 7–9 months with a live weight of 20–25kg. Prior to immunization, animals were climatized for two weeks, and body temperature and clinical examinations were performed daily. Blood and serum samples were collected once for assessing anti-BTV neutralizing antibodies before the experiment. Animals were maintained in accordance with the "Instructions for the maintenance of experimental animals and veterinary-sanitary rules in experimental biological isolators" inventory number 1069. All animal work was carried out in compliance with national guidelines on animal handling. The animal protocol used in this study was approved by the Committee on the Ethics of Animal Experiments of the Research Institute for Biological Safety Problems of the Science Committee of the Ministry of Education and Science of the Republic of Kazakhstan (Permit Number: 0110/13).

Purified antigen was used to immunize sheep and goats. Before immunization, animals were tested for the presence of BTV specific antibodies to no prior infection. Sheep and goats were inoculated subcutaneously with 1.0mL (300µg) of the antigen directly above the pre-scapular lymph nodes. After 14 days, a second subcutaneous immunization above the popliteal lymph nodes was carried out with 1mL solution containing a 3:7 ratio of 500µg of the antigen to Montanide adjuvant (ISA-71VG, Seppic, France). The addition of the adjuvant was carried out according to that previously described (Zhugunissov et al. 2018). During the observation period (21 days) post-second immunization, serum samples from sheep and goats were taken to detect BTV antibody levels every second day. Antibodies were detected using AGID and SNT. The immunization scheme is depicted in Fig. 1.

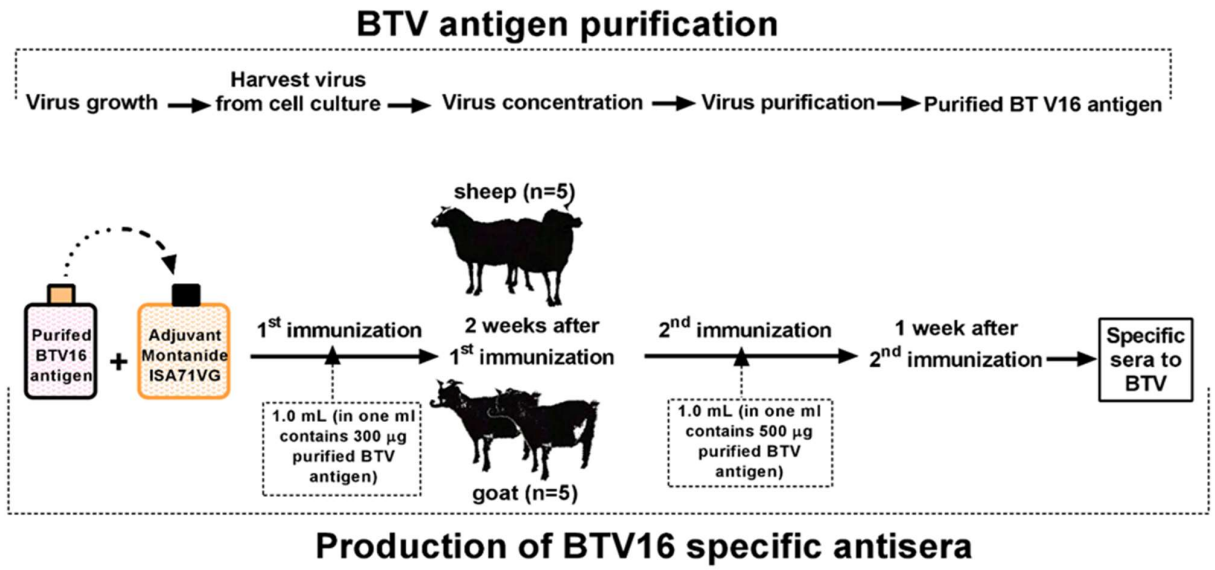

Fig. 1: Overview of the experimental procedure used in this study.

Table 1: Infectivity of BTV-16 after concentration

Virus serotype BTV16 sAgPassage #	Infectious activity, lg $TCID_{50}$/mL Initial	concentrate	The difference in titer between the initial and concentrated virus	P value
I passage	7.08±0.22	8.22±0.38	~1.14	>0.10
II passage	7.41±0.08	8.97±0.14	~1.56	<0.03
III passage	7.58±0.08	8.97±0.08	~1.39	≥0.05
IV passage	7.75±0.14	9.25±0.21	~1.50	<0.04
V passage	7.83±0.83	9.83±0.25	~2.00	<0.01

Evaluation of the effectiveness of viral separation from other proteins was carried out according to the formula (Logginov 1965):

$$\text{Viral clearance (\%)} = 100\% - (V_1 \times C_1)/(V_2 \times C_2) \times 100,$$

where: V_1 is the volume of the final material, C_1 is the concentration of the final material, V_2 is the initial volume, and C_2 is the starting material concentration.

SNT was performed to detect VNA titers according to the method described by Haig (1956) using BTV-16 serotype. Serotype-specific BTV-16-positive and negative antisera were used as control. Briefly, sera were diluted (1:2 TO 1:4096) in 96-well plates and VNA titers were estimated against 100TCID50 of BTV-16. Plates were incubated for 1h at 37°C and maintained at 4°C overnight. After incubation, 50µL of Vero cells suspension containing 2×105cells/mL was added to each well and plates were incubated for 4–7days at 37°C, 5% with CO2 atmosphere. Plates were screened for the presence of BTV induced CPE. Neutralization titers were determined as the inverse of sera dilution giving 50% neutralization end point.

AGID was used to determine BTV antigenicity, according to Pearson and Jochim (1979). BTV antibody response in sheep and goats was analyzed using GraphPad Prism Software, version 6.0 (GraphPad Software Inc, San Diego, CA, USA). The mean of the infected and control groups at specified time intervals was calculated and expressed as the mean ± standard error (SEM). Serological results and body temperature measurements between immunized and non-immune groups were analyzed using one-way ANOVA (analysis of variance) test. For all comparisons, differences were considered significant if $P<0.05$.

RESULTS and DISCUSSION

Higher titers of BTV were observed after 4–5 passages in Vero cells cultured in roller flasks. BTV titers were significantly higher ($P<0.03$) after four passages with titers ranged between 6.75 TO 8.00, log10TCID after 76hours of incubation (Fig. 2a). Antigenicity of BTV-16 was determined by AGID (Data not shown), and the antigenicity of BTV in Vero cells cultured in roller vessels was from 1:32 to 1:128. BTV only reacted with homologous antiserum. After concentration, BTV was viable, and we observed a significant increase of 2 logs in the virus yield (Table 1). Moreover, the antigenicity of the concentrate, as measured by AGID, was, on average, six orders (log2) of magnitude higher than the initial (Table 2).

Purification of BTV-16 by the combined freon/ ultracentrifugation method led to the production of 94.5 to 95.8% (w/vol) suspension of protein concentration after each step, respectively (Tab. 3). BTV yield was 5-8mg of protein from 1L of tissue culture media. Fig. 2c shows an electron micrograph of purified BTV16. After 3-4days post-immunization (dpi), a transient increase in body temperature was observed in both sheep and goats, with body temperatures returning to normal values by day five dpi. After 2-4days post-second immunization, animals exhibited another transient increase in body temperature (40.8±0.3°C) that lasted 48hours.

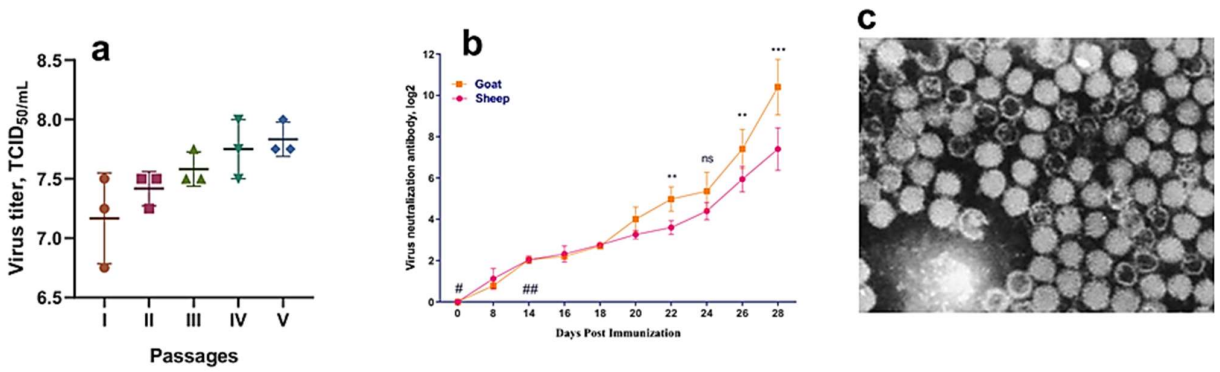

Fig. 2: (a) – Titers of BTV viral suspensions grown in Vero cells in roller vessels; (b) – dynamics of antibody formation against BTV in immunized animals (sheep, goats) for 28 days. (c) – electronic microscopy image of purified BTV16 at 100,000x magnification. Notes: (a) – Five BTV passages were carried out in Vero cell culture, and their infectious activity was measured in log10TCID/ml; (b) – Immunization of sheep and goats was carried out according to the scheme shown in Fig. 1. (#) -1st and (##) – 2nd immunization. (ns) – no significance. (**) – P≤0.05. (***) – P≤0.0001.

Table 2: Antigenicity of concentrated BTV-16

Virus serotype BTV16 sAg Passage #	Virus serotype	The antigen titer in the AGID, log2		The difference in titer between the initial and concentrated virus	P value
		Initial	concentrate		
I passage	BTV16 sAg (I passage)	5.0±0.11	11.3±0.13	~6.3	<0.0001
II passage	BTV16 sAg (II passage)	5.1±0.21	11.6±0.08	~6.5	<0.0001
III passage	BTV16 sAg (III passage)	5.1±0.05	10.5±0.11	~5.4	<0.004
IV passage	BTV16 sAg (IV passage)	6.2±0.13	12.1±0.11	~5.9	<0.0001
V passage	BTV16 sAg (V passage)	7.3±0.23	13.5±0.14	~6.2	<0.0001

Table 3: Purification of BTV16 by freon/ultracentrifugation method

Virus serotype BTV16 sAg Passage #	Protein concentration, mg/mL		Purification degree, %
	Initial	Concentrate	
I passage	4.7	7.6	~94.5
II passage	4.7	8.0	~95.8
III passage	4.8	8.2	~94.7
IV passage	4.9	8.4	~94.8
V passage	4.9	9.9	~95.5

Antibody responses to BTV16 in immunized sheep and goats were analyzed using VNT/SNT. The antibodies detected by VNT against BTV-16 in both sheep and goats were detected six dpi (Fig. 2b). However, in goats, neutralizing antibodies were detected significantly earlier after secondary immunization starting from 20 and 22dpi (P<0.05-P<0.0001) compared to sheep (Fig. 2b). Table 4 shows BTV-16 activity and specificity of sera obtained from immunized sheep and goats. BTV-16 antisera obtained from immunized sheep and goats showed low *in vitro* cross-reactivity with other serotypes BTV-4 and BTV-2. Interestingly, goats had a significant higher (P<0.05 to P<0.0001) BTV-16 antibody titer relative to sheep obtained after the second immunization 7.0 log2 to 13.0 log2 (average 10.8±2.28 log2).

Without a purified viral antigen, it is not possible to develop any diagnostically accurate test systems. For instance, an insufficient purification of viral proteins may lead to false-positive results in ELISA, such as HIV infection, due to nonspecific cellular detection (Weiss et al. 1985). Thus, the preparation of highly specific antisera requires the use of highly pure antigens (Lin et al. 2022).

Of all the methods used for viral concentration and partial purification on infected cell lines, the most effective is the precipitation method based on the use of PEG with a molecular weight of 6000 (Titov 1971). An increase in the antigenic activity of BTV viral suspensions after treatment with Freon-113 is probably due to either the removal of the "pseudo-envelopes" surrounding some viral particles or the removal of lipoprotein-type virus inhibitors (Xia et al. 2022).

In the study (Breard et al. 2021), the authors propose detection of BTV serotype 4-specific antibodies using a recombinant protein. The effectiveness of this alternative diagnostic method has been proven in the work. In a study (Ulisse et al. 2021), BTV-2 recombinant VP7 (BTV-2 recVP7), expressed in Spodoptera frugiperda (Sf9) cells using a baculovirus system, was produced and purified by affinity chromatography from the supernatant of infected cell culture. Thanks to the use of the supernatant, the authors obtained a high quantity of recombinant protein with high purity level by an easy one-step procedure.

Processing purified BTV preparations with chloroform, ether, and sodium deoxycholate led to a partial loss of its antigenic activity, which confirmed previously published results by Studdert (1965), indicating viral sensitivity to lipid solvents. Here, antibodies obtained in sheep against the BTV-16 showed a titer of 1:64 in AGID. The activity of goat antibodies against the BTV-16 serotype in AGID was 1:16 and 1:32, respectively. In summary, the use of freon for BTV-16 concentration followed by ultracentrifugation yielded a high concentration of viral antigens that, upon immunization, produced a high titer of viral-specific antibodies.

Table 4: Evaluation of BTV-16 antisera by AGID

Specific serum to BTV-16		nAg	BTV-16	BTV-4	BTV-2	PPRV	SPV
Sheep sera (n=5)	I	Nd	2.0±0.11 (1.0 – 3.0)	0.6±0.55 (0.0 – 1.0)	0.2±0.45 (0.0 – 1.0)	nd	Nd
	II	Nd	7.6±0.89 (7.0 – 9.0)	2.2±0.45 (2.0 – 3.0)	1.0±0.00 (1.0 – 1.0)	nd	Nd
Goat sera (n=5)	I	Nd	2.8±0.45 (2.0 – 3.0)	0.8±0.45 (0.0 – 1.0)	0.4±0.55 (0.0 – 1.0)	nd	Nd
	II	Nd	10.8 ±2.28 (7.0 – 13.0)	2.4±0.55 (2.0 – 3.0)	1.0±0.00 (1.0 – 1.0)	nd	Nd

Notes: I is 1st immunization, II is 2nd immunization, nd is not detected, nAg is normal antigen from cell culture. Data are shown on Mean±SD. The minimum and maximum values are shown in brackets.

Conclusion

We observed a moderate cross-reactivity of the BTV-16 antisera with BTV-2 and BTV-4. This phenomenon is explained by the presence of the VP7 polypeptide, a major protein localized on the surface of the virion's inner shell, which determines the serogroup specificity of the pathogen serotypes. Higher anti-BTV-16 activity was observed with goat antisera to enable the effective production of antibodies against the virus.

The technique described in this paper has significant advantages, such as simplicity, efficacy, and productivity; moreover, it does not require expensive equipment and is useful for large-scale production. Therefore, we used PEG-6000 to concentrate and purify BTV-16. Further, studies were conducted to purify the concentrated BTV-16. When determining a purification step, it is necessary to quickly and efficiently concentrate the virus from the available starting material. Thus, the two-fold addition of PEG-6000 at a final concentration of 5% (w/vol) resulted in an increased infectious virus titer (by log2) and increased antigenic activity (13 times). Thus, using a combined method of viral concentration followed by purification produced from 94.5 to 95.8% protein purification degree.

Author's Contribution

ZhK, ZhK, and MS designed and supervised the research. YeA and KZh assisted in the acquisition, analysis and interpretation of the data. MS wrote the manuscript. All authors have read and approved the final version of the manuscript.

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