Immunoaffinity purification of bluetongue virus group specific antibody using recombinant protein adsorbed to polystyrene wells

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ABSTRACT
Most of the common viral diseases can be diagnosed by serological assays and these assays mostly depend on the purity and quality of antibody used. Such specific antibodies can be generated by hybridoma technology. Alternatively, the virus-specific antibodies can be purified from polyclonal serum by immunoaffinity purification technique. Based on this immune affinity purification technique we have purified group-specific antibody against Bluetongue virus (BTV) using recombinant protein VP7 of BTV bound to polystyrene wells. Elution buffer consisting of 100 mM Glycine-HCl, pH 3.0 was found optimum for dissociation of the antibody from recombinant antigen and also to maintain the integrity of antigen. The reactivity of eluted antibody was tested in enzyme-linked immunosorbent assay (ELISA). The purified antibody will be useful in other serological assays like ELISA, fluorescent assay, and agar gel immunodiffusion (AGID) for Bluetongue disease diagnosis.

Key words: Bluetongue virus, ELISA, Group specific antibody, Immunoaffinity purification, Recombinant protein VP7.

INTRODUCTION
Bluetongue virus (BTV) belongs to genus Orbivirus with in the Reoviridae family causing disease in domestic and wild ruminants (Mertens et al., 2004). Till date, worldwide 27 serotypes of BTV have been reported which are transmitted amongst vertebrate hosts by Culicoides (Zientara et al., 2014). In India, 22 serotypes have been reported based on virus isolation and antibody detection (Chand et al., 2015). BTV genome is segmented double-stranded RNA (dsRNA) which codes for seven structural (VP1-VP7) and four nonstructural (NS1, NS2, NS3/NS3A and NS4) proteins (Belhouchet et al., 2011). The VP7 is the major structural protein of the cores of the virus and it is group specific antigenic determinants (Huismans and Erasmus, 1981). Serological test based on polyclonal antibody diagnostics like enzyme-linked immunosorbent assay (ELISA), complement fixation test (CFT) and agar gel immunodiffusion (AGID) are commonly used for the diagnosis of BTV (Afshar et al., 1989). These assays mostly depend on the purity and quality of antibody used.

Purification of antibodies in small scales in microtitre plate using synthetic peptide/recombination antigen has been tried in case of rubella (Pullen et al., 1986) Plasmodium falciparum (Brahimi et al., 1993) foot and mouth disease (Bayry et al., 1999) digoxin (Xu et al., 2010) and erythropoietin from human plasma (Mallorqui et al., 2010). In the present study, a method for efficient purification of group specific polyclonal antibody against BTV was optimized using recombinant VP7 protein adsorbed to polystyrene wells. The antibody was dissociated using different elution buffer and optimum elution buffer was selected based on the effect of various elution buffers on bound antibodies and on the adsorbed recombinant protein. The reactivity and specificity of the eluted antibodies were tested by sandwich ELISA (s-ELISA).

MATERIALS AND METHODS
Production of recombinant antigen and hyperimmune serum: pET32a plasmid carrying VP7 of bluetongue virus serotype 23 (BTV-23) was used for recombinant protein to bound with polystyrene well and protein was produced as the method described by Pathak et al. (2008). Hyperimmune serum (HIS) against whole virus and core particle of BTV was produced in rabbit and guinea pig by the method described earlier with modification (el Hussein et al., 1989). The HIS produced was used for immunoaffinity purification of group specific polyclonal antibody.

Immunoaffinity purification: The layout of the immunoaffinity purification technique is given in Fig. 1. The plate was divided into four parts (four well/parts). The 300 ng/well of recombinant protein VP7 (r-VP7; BTV-23) in carbonate bicarbonate buffer, pH 9.6 was dispensed in immuno module (MaxisorpNunc A/S, Roskilde, Denmark) and incubated at 37°C for 1h. The plates were washed three times with washing buffer (PBS containing 0.03% Tween-
20) and blocked by adding blocking buffer (3% skim milk powder and 2% gelatin in PBS). Then anti BTV-23 rabbit hyperimmune serum was added and incubated at 37°C for 1 h and washed thrice. To detect the presence of bound antibodies, anti-rabbit HRPO conjugate was added to the one of the well in each part and incubated at 37°C for 1 h. After final washing, substrate/chromogen mixture (hydrogen peroxide/orthophenylenediamine) was added; the reaction was stopped by 1 M H$_2$SO$_4$ and absorbance was measured at A$_{492}$. The bound antibodies in remaining wells were eluted separately with 200 µl each of the different elution buffers viz. Buffer A (4M MgCl$_2$), Buffer B (3M KSCN), Buffer C (5M LiCl) and Buffer D (100mM Glycine-HCl, pH 3.0). All the three sets of immunomodule were incubated separately at 37°C for 5, 15 and 30 minutes and supernatant were collected. Effect of elution buffer on the bound antibodies and the adsorbed recombinant protein was tested further by two steps ELISA. The one well of each treated well with elution buffer was reacted with anti-rabbit anti HRPO conjugate, to quantify the non-eluted antibodies. To the remaining wells after blocking with blocking buffer, added anti-BTV rabbit serum (diluted 1:1000) followed by HRPO conjugate to access the integrity of recombinant antigen. After washing substrate was added and absorbance was measured at A$_{492}$. The above procedure was also followed for the purification of antibody produced in the guinea pig.

Repeated antibody purification: After optimization of elution buffer and incubation time, the antigen bound to plate wells were tested for its integrity. After each elution, the wells were blocked with blocking buffer and treated with diluted polyclonal serum and the presence of bound antibodies was detected by respective anti-species HRPO conjugates. Purified eluted antibodies from each repeated elution were used as a capture and detection antibody in sandwich ELISA (Chand et al., 2009). The antigen coated strips were kept at 4°C for 3, 7, and 14 days to access the shelf life and integrity of the antigen coated strips. After each period of incubation, the polyclonal antibodies were allowed to react with coated recombinant protein. The bound antibody was detected by respective anti-species conjugate in one set of wells and repeated affinity purification of bound antibodies in remaining wells. Further, after elution integrity of coated antigen was checked as per above described procedure.

RESULTS AND DISCUSSION

The bound specific antibodies were eluted with different buffers (buffers A, B, C and D) at 5, 15 and 30 minutes as described. The efficiency of each buffer system for eluting the bound antibodies was evaluated by detecting the presence of left over antibodies by ELISA. Buffer D showed the higher efficiency of antibody elution as detected by a decrease in the A 492 value from 1.05 ±0.04 to 0.30±0.02 after 30 min elution while it was 0.45±0.04, 0.40±0.05 and 0.80±0.02 as against control value of 1.05 for buffer A, B and C respectively for the incubation period of 30 minutes. The efficiency of elution buffers on the dissociation of antibody and integrity of coated antigen were studied by two steps ELISA. In two steps ELISA, a positive reaction with anti-rabbit conjugate after elution, quantify
the non-eluted antibodies while positive reaction with anti-BTV antibodies followed by a conjugate reveals the integrity of the coated antigen. The sum of absorbance values of ELISA conducted for the integrity of coated antigen and quantification of non-eluted antibodies after elution was calculated for each buffer system. The resulting $A_{492}$ was compared with that of control taken as 100% and percentage efficiency of elution of each buffer was calculated. As shown in Fig.2 with buffer A (Fig.2 A), as taking % $A_{492}$ for control as 100% the % $A_{492}$ of eluted antibodies was 50.5% 56% and 57% after 5, 15 and 30 min of incubation respectively. With buffer B (Fig.2 B) 52%, 57% and 62% after 5, 15 and 30 min of incubation respectively. However with buffer C (Fig.2 C) the percentage of eluted antibodies was very low and it was 19%, 22% and 24% after 5, 15 and 30 min of incubation respectively. The Buffer D (Fig.2 D) showed highest elution efficiency 58%, 70.5% and 72% after 5, 15 and 30 min of incubation respectively without any adverse effect on the integrity of the adsorbed recombinant antigen. Therefore, buffer D (100 mM Glycine-HCl, pH 3.0) was used as a final elution buffer for further studies. There was a minor difference in elution efficiency between 15 and 30 min with buffer D, therefore 15 min was considered to be optimum and followed in the subsequent experiment.

For repeated use of the recombinant antigen coated wells for the purification of specific antibodies, three repetitive elution were carried out as per method described earlier. After each elution, the antibodies were collected to assay the presence of virus-specific antibody and the integrity of the coated antigen. Purified antibodies were used as coating and tracing antibody in-house developed sandwich ELISA. The OD value was 0.8±0.05 elution; however, after third elution there was a drastic reduction in OD value (i.e. ~0.4±0.04 as compared to control value of 1.10±0.05). This could be due to detachment of coated antigen as a result of repeated elution or decrease in the shelf life of antigen after storage. It is evident from above experiment that, the affinity purification could be carried out for a maximum of three times from the same antigen coated wells.

![Graphs showing elution efficiency of different buffers](image-url)

**Fig-2.** Effect of various elution buffers on the elution of bound antibodies and integrity of the bound recombinant antigen. The percentage of eluted and non-eluted antibodies were determined by two-step ELISA. A,B,C and D shows the elution pattern of various buffers A,B,C and D respectively.
The protein-coated polystyrene wells were kept at 4°C and self-life was studied at 3 and 7 and 14 days after incubation. The wells were allowed to react with anti-BTV antibody and immunoglobulins were eluted with buffer D. After 3 days the $A_{492}$ of control wells (ELISA for bound antibody) 0.80 against that of 1.05 of the freshly adsorbed strip as shown in Fig.3. However, after 7 days $A_{492}$ values decrease to 0.52 indicating the detachment or degradation of the protein. Further after 14 days, $A_{492}$ values decrease to 0.25. This indicates the loss of adsorbed protein over a period of incubation. Hence fresh antigen coated immune module is better for the elution of the antibodies. Antibody against BTV-1, 2,9,18 and 23 (serotype predominantly available in India) has been purified using optimized immunopurification technique. Sandwich ELISA was carried out for evaluating the utility of affinity purified antibodies. The s-ELISA was carried out by the method described earlier (Chand et al., 2009). In s-ELISA, the $A_{492}$ value for the eluted antibody fraction was 1.0 ±0.2 indicating the presence of antibody in eluted fraction.

Specificity of BTV serological tests largely depends on the quality of antibodies used in the test. Production of group specific antibodies using hybridoma technique is superior but several limitations like screening, laborious and maintenance of hybridomas. An alternative of this is to the selective purification of the group specific antibodies from antiserum using antigen affinity column. Several affinity matrices like CNBr coupled agarose and Ni-NTA agarose bound protein have been used to achieve purity of the serum (Jun et al., 1994). Antigen adsorbed polystyrene wells have been tried and used for small-scale purification of monospecific antibodies to antigens from Plasmodium falciparum (Brahimi et al., 1993). Recombinant protein adsorbed to polystyrene well has been also used for the type specific FMDV antibody purification (Bayry et al., 1999). An erythropoietin (EPO) specific immunoaffinity microplate was used for purification of human erythropoietin (hEPO) from human plasma sample (Mallorqui et al., 2010). An ELISA based elution procedure was used for dissociation of digoxin-antibody complexes in immunoaffinity chromatography (Xu et al., 2010). In order to purify the group-specific antibody for BTV, we used recombinant protein VP7 that carries group specific epitope. This is probably the first report where recombinant protein was used for purification of antibodies against BTV. In this study elution buffers were selected based on acidic (glycine-HCl) and chaotropic property of the agents. Buffers which dissociate antigen-antibody complexes due to their chaotropic nature at neutral or slightly acidic pH were preferred. The effect of each elution buffer on antibodies bound to recombinant as well as the integrity of coated antigen was studied. As one step indirect ELISA is incapable to assess the efficiency of antibody elution and integrity of coated antigen after treatment with elution buffer, a two-step procedure was followed to validate the effectiveness of each buffer selected. Each buffer system required varying time periods to attain maximum elution. Buffer A and B was found effective after 30 min while buffer D showed suitable elution efficiency after 15 min and it was marginally higher after 30 min. Buffer C was least effective even after 30 min of incubation. Buffer D (100 mM glycine-HCl, pH 3.0) was found to be most suitable to maintain the integrity of coated antigen after effective elution of bound antibody.

VP7 is a group specific antigen and antibody against all BTV serotype can bind with r-VP7 antigen. Therefore, the antibody against all-serotype can be purified using optimized immunoaffinity purification technique. In the present study, an antibody against BTV-1, 2,9,18 and 23 serotypes has been purified by immunoaffinity purification. The antigen-coated strips could be used for antibody purification twice repeatedly with a slight decrease in elution yield. Subsequent use reduces the antibody yield drastically. This could be due to degradation or detachment (or both) of antigen from the wells on repeated elution and washing. In the present experiment, there was a 25 % decrease in the efficiency of antibody elution when the strips were used second time (3 days) of storage at 4°C. However, there is decrease in efficiency up to 50 % and 75 % after third (7 days) and fourth (14 days) elution respectively.

Our studies showed that recombinant VP7 protein could be used for immunoaffinity purification of group-specific antibody. For the production of small scale group specific BTV antibody the use of affinity purification technique using recombinant protein is simple and cost effective.
effective and purified BTV specific antibody will be used in the various diagnostic assay of bluetongue. We have used the purified antibody in-house developed s-ELISA for detection of BT antigen, in which earlier crude antibody was used.

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REFERENCES


