

# Identification of two novel BTV16-specific B cell epitopes using monoclonal antibodies against the VP2 protein

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Received: 8 January 2013 / Revised: 12 February 2013 / Accepted: 13 February 2013 / Published online: 7 March 2013  
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**Abstract** The VP2 protein of bluetongue virus (BTV) is an important structural protein and is the principal antigen responsible for BTV serotype specificity. In this study, we mapped the reactivity of two BTV16-specific monoclonal

**Electronic supplementary material** The online version of this article (doi:10.1007/s00253-013-4779-z) contains supplementary material, which is available to authorized users.

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antibodies (MAbs) and identified two novel serotype-specific linear B cell epitopes on the BTV16 VP2 protein. By screening a series of peptides derived from the BTV16 VP2 protein and expressed as mannose-binding protein fusions, we determined that the linear epitopes recognized by the VP2-specific MAbs 3 G10 and 2B4 were located within the peptides  ${}_{34}\text{EWSGHVDVTEIPNRRMF}_{49}$  and  ${}_{540}\text{KNEDPYVKRTVKPIRA}_{555}$ , respectively. To define the minimal region required for antibody binding within these peptide regions, a series of progressively shorter peptides were synthesized and evaluated for 3 G10 and 2B4 binding. This work defined the motifs  ${}_{34}\text{EWSGHVDVTEIPNRRMF}_{49}$  and  ${}_{543}\text{DPYVKRTVK}_{555}$  as the minimal linear peptides required for 3 G10 and 2B4 binding, respectively. Alignment of amino acid sequences from a number of BTV16 strains isolated from different regions indicated that these two epitopes are highly conserved among BTV16 strains. Furthermore, these two epitopes are not conserved among other BTV serotypes or prototype members of the genus *Orbivirus* in the family *Reoviridae*, as shown by sequence alignments. The MAb reagents and linear epitopes defined here provide the basis for the development of epitope-based serotype-specific differential diagnostic tools and may be useful in the design of epitope-based vaccines.

**Keywords** Bluetongue virus · VP2 protein · Serotype-specific monoclonal antibody · Epitopes

## Introduction

Bluetongue virus (BTV) is the etiological agent of bluetongue disease, a noncontagious, insect-transmitted disease of sheep and some species of wild ruminants (Boone et al. 2007). Due to the potential rapid spread of BTV and the

threat to the international trade of livestock and livestock products, bluetongue disease was listed as 1 of 16 diseases formerly classified in list A by the Office International Des Epizooties.

BTV is the prototype member of the genus *Orbivirus*, family *Reoviridae*. The BTV genome consists of ten double-stranded RNA genome segments that collectively encode seven structural (VP1 to VP7) and five nonstructural (NS1, NS2, NS3, NS3A, and NS4) proteins (Roy 1989, 1992; Ratnien et al. 2011). The inner capsid or core is composed of two major proteins (VP3 and VP7) and three minor proteins (VP1, VP4, and VP6) and contains the viral genome (Roy 1992, 2005). VP2 and VP5 proteins form a continuous layer over the inner capsid in which multimers of VP2 (dimers and/or trimers) are layered upon a VP5 scaffold. The resulting structures protrude 3 nm beyond the main body of the virion particle and are responsible for the adsorption and entry of BTV into mammalian cells, are responsible for hemagglutination, and are the target of neutralizing antibodies and are, therefore, the primary determinants of serotype specificity (Roy 2008; Hassan and Roy 1999; Bonneau et al. 2001; Zhang et al. 2010). There is considerable genetic and antigenic variation within the BTV serogroup, with 26 distinct serotypes and considerable strain variation within each serotype. As a result, there is little or no immune cross-protection among different BTV serotypes and it has been difficult to develop a useful multivalent vaccine. The development of serotype-specific differential diagnostic approaches is currently needed to facilitate the control of BTV.

Virus infection elicits the production of antibodies against viral proteins. Defined B cell epitopes, defined as regions on the surface of the native antigen that are recognized by B cell receptors or specific antibodies, have emerged as promising targets for vaccines and diagnostic reagents (Tian et al. 2012). Specifically, the identification of serotype-specific epitopes on various proteins of the BTV serogroup would have considerable diagnostic value. While the VP2 protein is a major determinant of viral serotype, serotype-specific epitopes on the VP2 protein of BTV16 have not been reported. We developed two serotype-specific monoclonal antibodies (MAbs) that recognized the VP2 protein of BTV16. Here, we define the linear peptide epitopes recognized by these two MAbs using a series of partially overlapping peptides derived from the BTV16 VP2 protein amino acid sequence to map MAb reactivity. These MAbs and their defined linear epitopes may support the development of serotype-specific differential diagnostic tools and marker vaccines which enable differentiation of infected from vaccinated animals.

## Materials and methods

### Cell lines and virus

Baby hamster kidney-21 cells (BHK-21 cells) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere with 5 % CO<sub>2</sub> at 37 °C. All culture media were supplemented with 10 % heat-inactivated fetal bovine serum (GIBCO, Invitrogen) and antibiotics (0.1 mgml<sup>-1</sup> of streptomycin and 100 IUml<sup>-1</sup> of penicillin). Viruses from each BTV serotype (BTV1–24), Ibaraki virus (IBAV), and Chuzan virus (CV) were acquired from the State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences and were propagated in our laboratory using standard techniques. The GenBank accession number of BTV1 (strain SZ97/1) and BTV16 (strain BN96/16) is JN848767 and JN671915, respectively, and the remaining viruses have yet not been sequenced. A BTV16-reactive polyclonal antiserum was produced by infecting the sheep experimentally with BTV16 strain BN96/16.

### Expression and characterization of recombinant BTV16 VP2 protein

Recombinant VP2 protein was prepared using the Bac-to-Bac<sup>®</sup> Baculovirus Expression System according to the manufacturer's instructions (Invitrogen). Briefly, the full-length BTV16 VP2 coding sequence was amplified using the following primer pair: BTV16 VP2-*EcoRI*-22-40 (5'-ACGAATTCATGGAGGAGCTAGTTATACC-3') and BTV16 VP2-*SalI*-2901-2880 (5'-CCGTCGACTTAAATA TTTAGAAGCTTCGTG-3'). Primers incorporated restriction sites (italicized) to facilitate cloning of the amplified VP2 sequence into the pFast-Bac<sup>™</sup> vector. The recombinant pFastBac<sup>™</sup> vector was transformed into competent DH10Bac<sup>™</sup> *Escherichia coli*, which were subsequently plated on triple antibiotic LB plates (kanamycin, gentamicin, and tetracycline) with halogenated indolyl- $\beta$ -galactoside (BluoGal; Invitrogen). Bacmid DNA was recovered from transformed colonies and verified via polymerase chain reaction. Recombinant baculoviruses were generated by using the Cellfectin<sup>®</sup> II Reagent to transfect Sf9 cells with recombinant bacmid. Recombinant protein was purified by Ni-nitrilotriacetic acid affinity chromatography according to the manufacturer's instructions (Qiagen). The recombinant VP2 protein was evaluated by Western blot (WB) analysis using a BTV16-reactive polyclonal antiserum as a primary antibody source along with a donkey antisheep IgG (whole molecule)-peroxidase antibody (Sigma) as a secondary antibody.

## Production and characterization of monoclonal antibodies

BTV16 VP2-reactive MAbs were generated as follows. Briefly, 6-week-old female BALB/c mice were immunized with 100 µg per mouse purified recombinant BTV16 VP2 protein mixed with an equal volume of complete Freund's adjuvant (Sigma, St. Louis, MO, USA). Two booster injections containing purified BTV16 VP2 in an equal volume of Freund's incomplete adjuvant were given at 2-week intervals. Three days after the final booster, splenocytes were fused with SP2/0 myeloma cells using polyethylene glycol (PEG 4000; Sigma). The hybridoma cells were plated in 96-well plates and cultured in hypoxanthine–aminopterin–thymidine (HAT) selection medium (DMEM containing 20 % fetal bovine serum, 100 gml<sup>-1</sup> streptomycin, 100 IUml<sup>-1</sup> penicillin, 100 mM hypoxanthine, 16 mM thymidine, and 400 mM aminopterin). After 5 days, the medium was removed and replaced with hypoxanthine–thymidine (HT)-DMEM medium (DMEM containing 20 % fetal bovine serum, 100 gml<sup>-1</sup> streptomycin, 100 IUml<sup>-1</sup> penicillin, 100 mM hypoxanthine, and 16 mM thymidine). After selection in HAT or HT media, hybridoma supernatants were screened for reactivity and specificity by indirect enzyme-linked immunosorbent assay (ELISA), WB, and indirect immunofluorescence assay (IFA) as previously described (Wang et al. 2012a, b). This approach yielded two BTV16 VP2-reactive MAbs, which were named 3 G10 and 2B4.

## Polypeptide design and expression

To express a set of 87 partially overlapping peptides derived from the amino acid sequence of the BTV16 VP2 protein (Yang et al. 2011), we synthesized pairs of complementary oligonucleotides encoding each 16-amino-acid-long peptide sequence (Table S1). Oligonucleotides were designed so that the adjacent peptides in the series had five amino acid residues in common (e.g., the first peptide in the series corresponded to amino acids 1–16, while the second peptide in the series corresponded to amino acids 12–27). The oligonucleotide pairs were annealed to one another and cloned into the *EcoRI* and *SalI* sites of pMAL<sup>TM</sup>-C4x, which places the encoded VP2-derived peptide in frame with mannose-binding protein (MBP) to produce MBP-fused polypeptides. MBP-fused polypeptides in the series were named MBP-VP2-1 to MBP-VP2-87. The recombinant plasmids were transformed into *E. coli* BL21 (DE3; Novagen, USA). Transformed cells were grown in LB/Amp medium (1 % tryptone, 0.5 % yeast extract, 1 % NaCl; 0.1 mg/ml ampicillin) to an OD<sub>600</sub> of approximately 0.5–0.7. Isopropylthio-β-galactoside was added to a final concentration of 0.5 mM (Pharmacia Biosciences) to induce recombinant protein expression and the cells were agitated for an additional 6 h at 37 °C. Bacteria were pelleted at 9,000×g

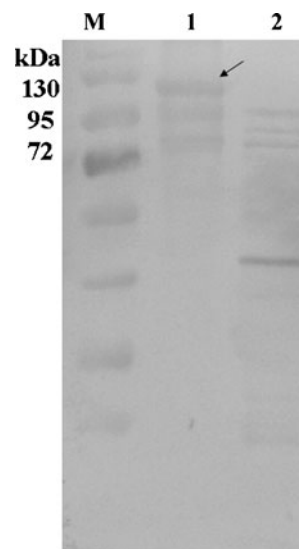
for 10 min at 4 °C and lysed. Polyacrylamide gel electrophoresis and WB was carried out to characterize the production of each MBP-fused recombinant polypeptide.

## Epitope identification

Two BTV16 VP2-reactive MAbs, 2B4 and 3 G10, were screened against the MBP-fused polypeptide series by indirect ELISA and WB. The ELISA was performed using the expressed MBP-fused polypeptides as coating antigen. The MBP tag without a fused polypeptide and a BTV VP7-specific MAb (clone IB7) were used as controls. The initial screen of the 87 overlapping VP2-derived peptides identified 2 regions of VP2 on which to focus a more refined mapping of the MAb-binding epitopes. To define the minimal linear peptide epitope recognized by MAb 2B4 and 3 G10, we synthesized and screened by ELISA a series of progressively truncated polypeptides based on the results of the initial screen.

## Homology analysis

To evaluate the conservation of the two identified linear VP2 epitopes among BTV16 isolates, we aligned sequences corresponding to the region encompassing the 2B4 and 3 G10 peptide epitopes from a number of BTV16 strains isolated from different geographic areas using the DNAMAN software



**Fig. 1** Recognition of recombinant VP2 by a BTV16-reactive polyclonal antiserum. Samples collected during production of recombinant VP2 proteins were analyzed by WB for the presence of VP2 protein using a BTV16-reactive polyclonal antiserum. Lane 1 insect cell lysates following infection with recombinant baculovirus encoding the BTV16 VP2 protein, lane 2 insect cell lysates following infection with wild-type baculovirus, lane M PageRuler<sup>TM</sup> Prestained Protein Ladder

(Lynnon BioSoft Inc., USA). Alignments were also performed between the defined epitopes and the corresponding regions of BTV serotypes 1–26 and prototype members of the genus *Orbivirus* in the family *Reoviridae* to evaluate potential epitope conservation.

The reactivity of MAbs with the corresponding epitope regions from different BTV16 strains

Analysis of the conservation of the two identified linear VP2 epitopes among BTV16 isolates using amino acid alignments identified some BTV16 isolates in which the linear epitopes were not 100 % conserved. The corresponding epitope regions of different BTV16 strains that were not identical to the two identified BTV16 VP2 epitopes were synthesized and tested for reactivity with the BTV16 VP2-reactive MAbs 3 G10 and 2B4 by indirect ELISA. The GenBank accession number of BTV16 VP2 used in this paper is no. JN671907.

## Results

### Expression and characterization of recombinant BTV16 VP2 protein

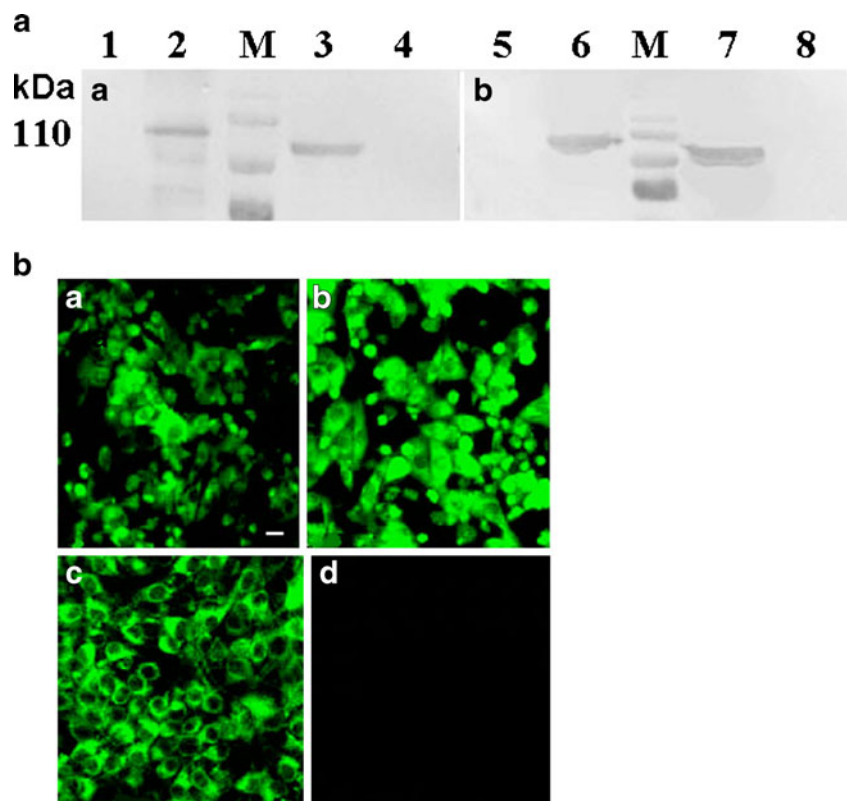
Recombinant BTV16 VP2 protein was expressed and purified using a baculovirus expression system. WB analysis

showed the recombinant protein migrated with the expected molecular weight and was recognized by a BTV16-reactive polyclonal sheep antiserum (Fig. 1, lane 1). Therefore, the recombinant VP2 protein produced in insect cells maintained epitopes that are recognized by antibodies elicited during BTV16 infection of sheep and was a suitable antigen preparation for immunization and hybridoma screening.

### Production and characterization of monoclonal antibodies

We used recombinant BTV16 VP2 protein to immunize mice in order to generate BTV VP2-reactive MAbs. Two clonal hybridoma cell lines that produced BTV16 VP2-reactive MAbs were identified by indirect ELISA and named 3 G10 and 2B4. MAb 3 G10 and 2B4 reacted with recombinant BTV16 VP2 protein expressed in insect cells (Fig. 2a, lanes 2 and 6) and native BTV16 VP2 protein expressed during BTV16 infection of BHK-21 cells (Fig. 2a, lanes 3 and 7) by WB. Next, the reactivity of each MAb against different BTV serotype viruses was evaluated. MAb reactivity was assessed by an IFA against BHK-21 cells infected with BTV serotype viruses, IBAV, CV. MAb 3 G10 and 2B4 reacted only with BTV16-infected cells and did not cross-react with cells infected with IBAV, CV, or any other BTV serotype tested (Fig. 2b, and data not shown).

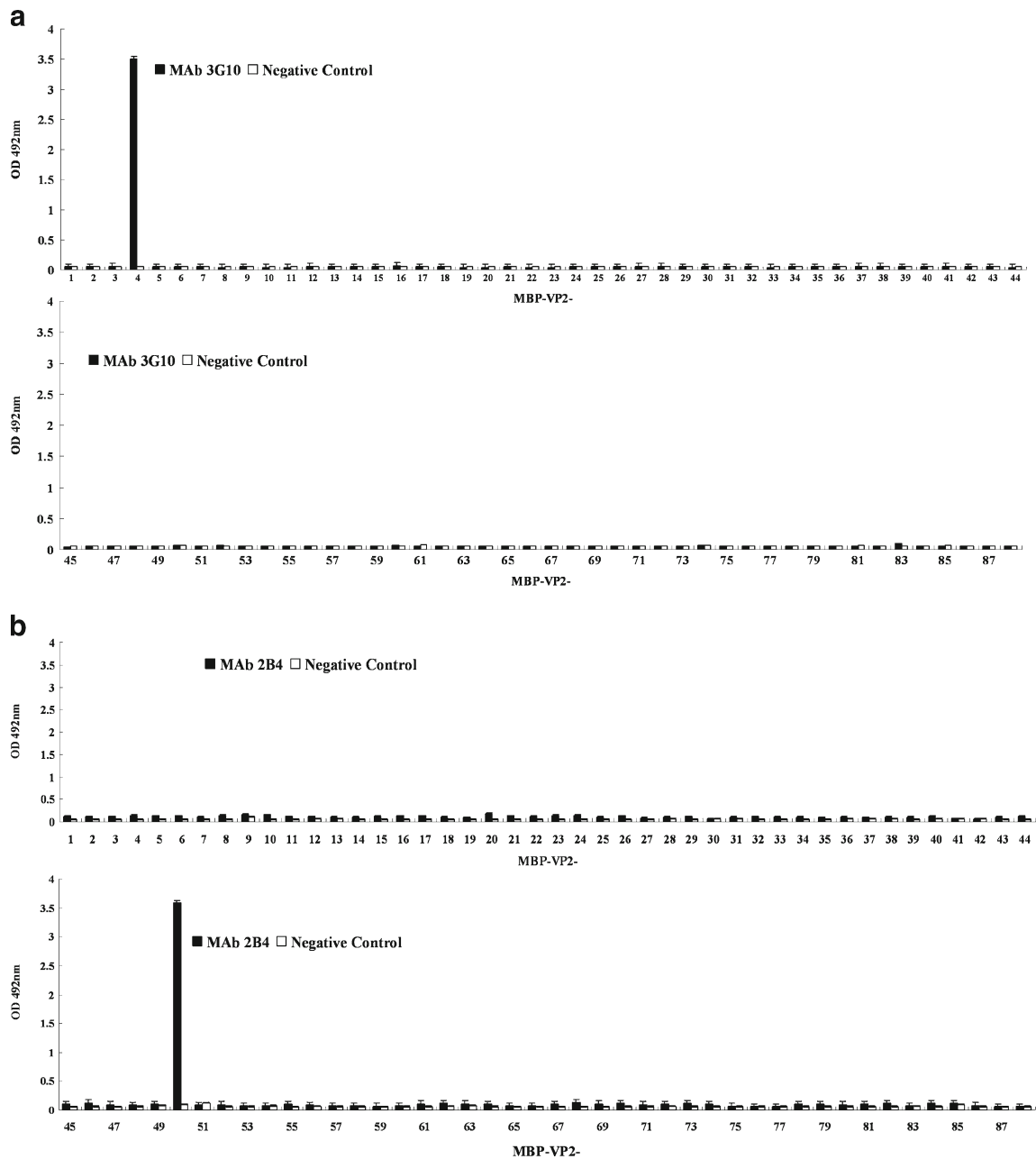
**Fig. 2** MAbs 3 G10 and 2B4 recognize BTV16 VP2 protein. **a** The reactivity of MAb 3 G10 (*a*) and 2B4 (*b*) was evaluated by WB against insect cell lysates following infection with recombinant baculovirus encoding the BTV16 VP2 protein (*lanes 1 and 5*), purified recombinant BTV16 VP2 protein (*lanes 2 and 6*), BTV16-infected BHK-21 cell lysates (*lanes 3 and 7*), and uninfected BHK-21 cell lysates (*lanes 4 and 8*). *Lane M* PageRuler™ Prestained Protein Ladder. **b** BTV16-infected BHK-21 cells were evaluated by IFA for reactivity with MAb 3 G10 (*a*), 2B4 (*b*) (*bar*, 10 μm), a BTV16-reactive polyclonal antiserum (*c*), and a negative control serum (*d*)



### Identification of BTV16 VP2 epitopes recognized by MAbs 3 G10 and 2B4

We next evaluated MAb 3 G10 and 2B4 reactivity against a series of 87 partially overlapping peptides derived from the amino acid sequence of the BTV16 VP2 protein in order to map antibody-binding epitopes. Each MBP-fused peptide in the VP2 peptide series was successfully expressed in *E.*

*coli* and migrated with the expected molecular weight (data not shown). MAb 3 G10 recognized one peptide in the series (MBP-VP2-4), corresponding to the sequence  ${}_{34}\text{EWSGHDVTEIPNRRMF}_{49}$  of the BTV16 VP2 protein (Fig. 3a). Similarly, MAb 2B4 reacted with one peptide in the series (MBP-VP2-50), corresponding to the sequence  ${}_{540}\text{KNEDPYVKRTVKPIRA}_{555}$  of the BTV16 VP2 protein (Fig. 3b). Consistent with these results, MAb 3 G10



**Fig. 3** Identification of the BTV16 VP2 linear peptide epitopes recognized by MAb 3 G10 and 2B4. A set of 87 partially overlapping peptides derived from the amino acid sequence of the BTV16 VP2 protein was screened by indirect ELISA to identify linear peptide epitopes recognized by MAb 3 G10 and 2B4. **a** Screening of MAb

3 G10 against the VP2 peptide series. **b** Screening of MAb 2B4 against the peptide series. An anti-BTV VP7 MAb was used as a negative control antibody, and the MBP tag with no polypeptide fusion was used as a negative control protein. *Error bars* reflect the results of three experimental repeats

recognized the MBP-VP2-4 peptide (Fig. 4, lane 1) and MAb 2B4 recognized the MBP-VP2-50 peptide (Fig. 4, lane 3) by WB, but neither antibody reacted with the MBP tag alone (Fig. 4, lanes 2 and 4).

We next sought to identify the minimal linear peptide epitopes in the BTV16 VP2 protein recognized by MAb 3 G10 and 2B4. A panel of truncated polypeptides were synthesized in which amino acids were progressively deleted from the amino and/or the carboxy terminus of the 16-amino-acid peptides recognized by 3 G10 and 2B4 in Fig. 3 ( ${}_{34}\text{EWSGHDVTEIPNRRMF}_{49}$  and  ${}_{540}\text{KNEDPYVKRTVKPIRA}_{555}$ , respectively). The deletion of a single residue from either the carboxy or amino terminus of the VP2-4 peptide recognized by 3 G10 resulted in reduced antibody binding, and the antibody binding was completely abrogated when a single residue was deleted from either the carboxy or amino terminus of the VP2-4 peptide (Fig. 5a). Therefore,  ${}_{34}\text{EWSGHDVTEIPNRRMF}_{49}$  is the minimal linear epitope required for optimal 3 G10 binding (Fig. 5a). In contrast, a number of amino acid residues could be deleted from both the carboxy and amino termini of the VP2-50 peptide without any detectable reduction in 2B4 antibody binding (Fig. 5b). Further removal of amino acids from the peptide sequence  ${}_{543}\text{DPYVKRTVK}_{551}$  resulted in a complete abrogation of 2B4 antibody binding, defining  ${}_{543}\text{DPYVKRTVK}_{551}$  as the minimal linear epitope recognized by 2B4 (Fig. 5b).

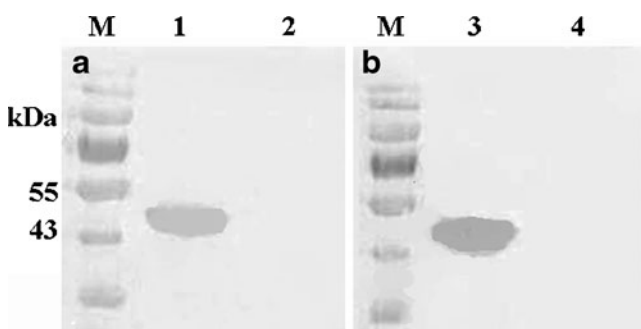
#### Homology analysis

We then evaluated whether the two BTV16 VP2 minimal linear epitopes were conserved among BTV16 strains isolated from different regions. The amino acid sequences corresponding to the region encompassing these two BTV16 VP2 minimal linear epitopes from a number of

BTV16 strains were identified and aligned (Tables 1 and 2). Both linear epitopes were highly conserved among the different BTV16 strains. When regions aligning with the MAb 3 G10 epitope  ${}_{34}\text{EWSGHDVTEIPNRRMF}_{49}$  were evaluated among the different BTV16 strains, we found two strains containing an amino acid substitution (Table 1). The Japanese BTV16 strain (accession number AB686213) had a lysine at amino acid position 46 in place of an arginine, and the Australian BTV16 strain (accession number JQ086232) had a serine at amino acid position 45 in place of the asparagine. The 3 G10 epitope  ${}_{34}\text{EWSGHDVTEIPNRRMF}_{49}$  was entirely conserved within the Mediterranean (accession number DQ191259) and European (accession number AM773701) BTV16 strains (Table 1). Similarly, the MAb 2B4 epitope  ${}_{543}\text{DPYVKRTVK}_{551}$  was well-conserved among the BTV16 strains evaluated. The Australian BTV16 strain harbored an isoleucine in place of a valine at amino acid position 550, and the Japanese BTV16 strain had an arginine in place of a lysine residue at amino acid position 551. The minimal linear epitope  ${}_{543}\text{DPYVKRTVK}_{551}$  was totally conserved within the Mediterranean and European BTV16 sequences (Table 2).

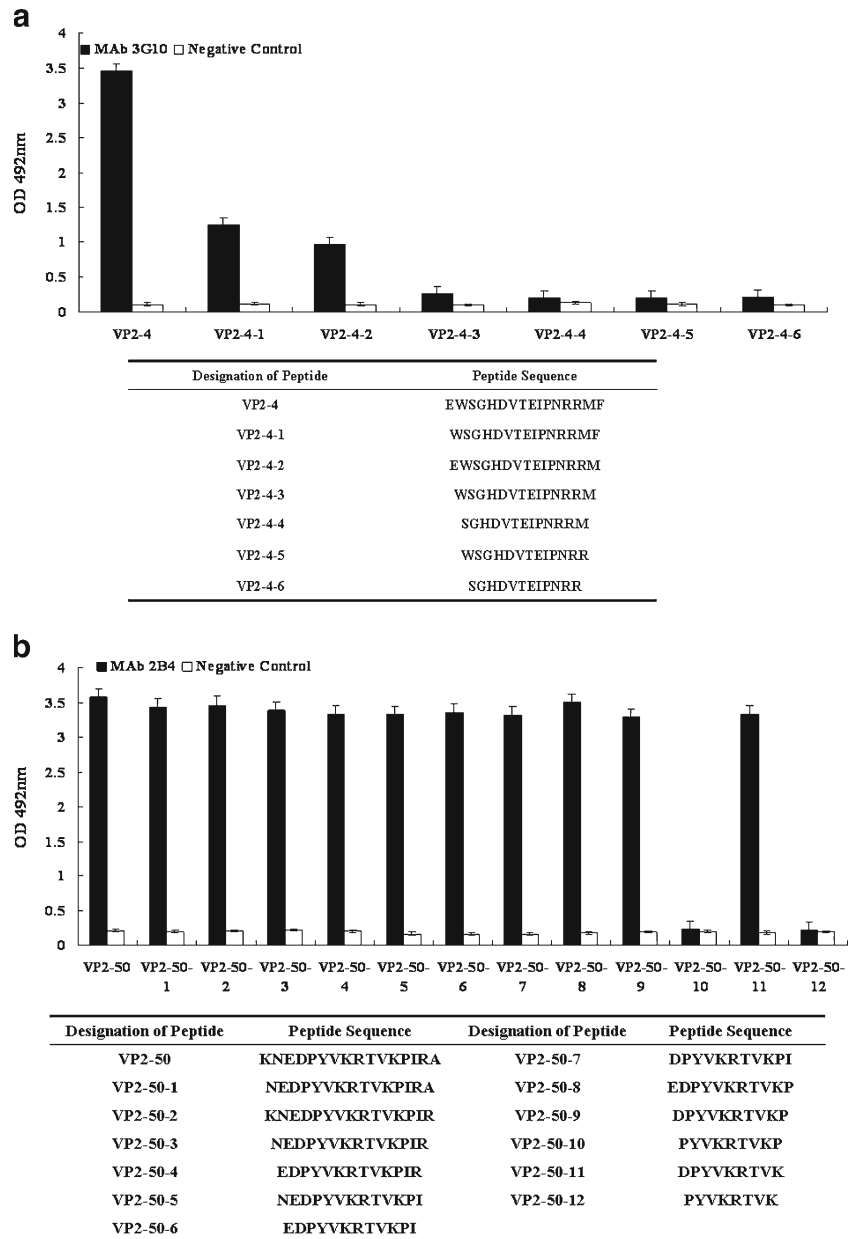
We next tested whether the MAbs 3 G10 and 2B4 recognized peptides containing the single amino acid substitutions in the corresponding epitope regions for some BTV16 isolates as described previously. Peptides with the single amino acid substitutions were synthesized and tested for reactivity with MAbs 3 G10 and 2B4 by indirect ELISA. The peptides EWSGHDVTEIPNRRMF and EWSGHDVTEIPSRRMF, corresponding to the epitope regions of the Japanese and Australian BTV16 isolates, showed strong reactivity with MAb 3 G10 despite the amino acid substitutions (Fig. 6a). The peptide DPYVKRTVR, corresponding to the epitope region of the Japanese BTV16 isolate, was still recognized by MAb 2B4. In contrast, 2B4 recognition the peptide DPYVKRTIK, corresponding to the epitope region of the Australian BTV16 isolate, was strongly reduced (Fig. 6b).

Amino acid alignments were also performed to evaluate the conservation of the two identified epitopes among different BTV serotypes and prototype members of the genus *Orbivirus* in the family *Reoviridae*. The MAb 3 G10 epitope was very poorly conserved among other BTV serotype viruses (Fig. 7a) and prototype members of the genus *Orbivirus* in the family *Reoviridae* (Fig. 7b). Similarly, the MAb 2B4 epitope was also poorly conserved among other BTV serotype viruses (Fig. 7c) and prototype members of the genus *Orbivirus* in the family *Reoviridae* (Fig. 7d). Collectively, these data show that the epitopes recognized by MAb 3 G10 and 2B4 are well-conserved among BTV16 isolates, but are not conserved among other BTV serotypes or related orbiviruses.



**Fig. 4** MAbs 3 G10 and 2B4 recognize their identified peptide epitope by WB. Reactivity of MAb 3 G10 (a) was assessed against the recombinant MBP-VP2-4 polypeptide ( ${}_{34}\text{EWSGHDVTEIPNRRMF}_{49}$ , lane 1) and the MBP tag alone (lane 2). Reactivity of MAb 2B4 (b) was assessed against the recombinant MBP-VP2-50 polypeptide ( ${}_{540}\text{KNEDPYVKRTVKPIRA}_{555}$ , lane 3) and the MBP tag alone (lane 4). Lane M PageRuler™ Prestained Protein Ladder

**Fig. 5** Progressively truncated peptides define the minimal linear epitope recognized by MAb 3 G10 and 2B4. MAbs 3 G10 (a) and 2B4 (b) were screened against a series of truncated peptides that had amino acid residues progressively deleted from the amino and/or carboxy termini to determine the minimal linear peptide sequence required for MAb binding. The name and sequence of the peptides used are included below the figure. A VP7-specific MAb was used as a negative control. Error bars reflect the results of three experimental repeats



**Discussion**

In the last decade, BTV has spread extensively to new areas and raised concerns regarding animal health, the farming

industry, and international trade (Purse et al. 2005; Wilson and Mellor. 2009). There are currently 26 distinct BTV serotypes. Each BTV serotype induces humoral immune responses that are generally not cross-reactive, thereby having

**Table 1** Amino acid sequence alignments of two BTV16 VP2 minimal linear epitopes among BTV16 strains isolated from different regions

| BTV16 strain                       | Epitope sequence <sup>a</sup> |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|------------------------------------|-------------------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| VP2_BTV16 (JN671907) China         | E                             | W | S | G | H | D | V | T | E | I | P | N | R | R | M | F |
| VP2_BTV16 (AB686213) Japan         | –                             | – | – | – | – | – | – | – | – | – | – | – | K | – | – | – |
| VP2_BTV16 (DQ191259) Mediterranean | –                             | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| VP2_BTV16 (AM773701) European      | –                             | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| VP2_BTV16 (JQ086232) Australian    | –                             | – | – | – | – | – | – | – | – | – | – | S | – | – | – | – |

<sup>a</sup> Dashes indicate identical bases

**Table 2** Amino acid sequence alignments of two BTV16 VP2 minimal linear epitopes among BTV16 strains isolated from different regions

| BTV16 strain                       | Epitope sequence <sup>a</sup> |   |   |   |   |   |   |   |   |   |
|------------------------------------|-------------------------------|---|---|---|---|---|---|---|---|---|
| VP2_BTV16 (JN671907) China         | D                             | P | Y | V | K | R | T | V | K |   |
| VP2_BTV16 (AB686213) Japan         | –                             | – | – | – | – | – | – | – | – | R |
| VP2_BTV16 (DQ191259) Mediterranean | –                             | – | – | – | – | – | – | – | – | – |
| VP2_BTV16 (AM773701) European      | –                             | – | – | – | – | – | – | – | – | – |
| VP2_BTV16 (JQ086232) Australian    | –                             | – | – | – | – | – | – | – | I | – |

<sup>a</sup>Dashes indicate identical bases

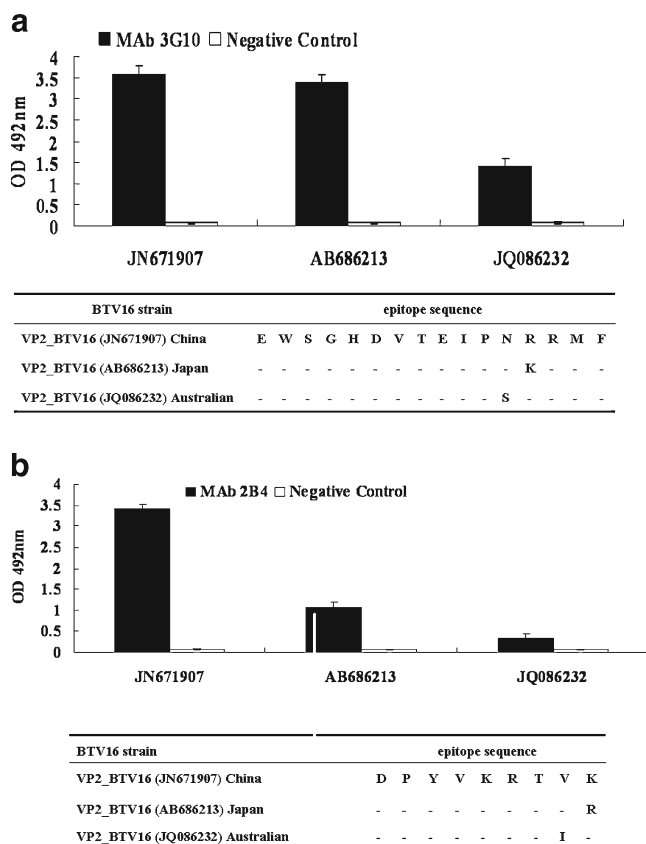
an impact on BTV vaccination strategies. The development of serotype-specific differential diagnostic techniques is needed to facilitate BTV control measures. To address this need, we focused on the VP2 protein. VP2 is the principal antigen responsible for BTV serotype classification and is the primary target of the neutralizing antibodies that confer protection against virulent virus challenge. A detailed analysis of the antibody epitopes within the VP2 protein would promote our understanding of BTV-specific immunity and the development of epitope-based marker vaccines and diagnostic tools for BTV (Peng et al. 2008). In this study, we developed two BTV16-reactive MAbs that recognized BTV16 but did not react with other BTV serotypes, IBAV or CV, and used them to map linear epitopes on the BTV16 VP2 protein.

The MAbs 3 G10 and 2B4 recognized recombinant BTV16 VP2 protein expressed in insect cells using the Bac-to-Bac<sup>®</sup> Baculovirus Expression System and native BTV16 VP2 protein expressed as a result of BTV16 infection of BHK-21 cells by WB. That antibody recognition of VP2 was maintained in WB suggested that both MAbs recognized linear peptide epitopes within the VP2 protein. We identified the region of VP2 recognized by each MAb by screening a set of 87 partially overlapping peptides derived from the amino acid sequence of the BTV16 VP2 protein, expressed as MBP-fused polypeptides (Sun et al. 2012). This screen identified a region of BTV16 VP2 recognized by MAb 3 G10 and a different region recognized by MAb 2B4.

Having identified a linear region of VP2 recognized by each MAb, we synthesized progressively truncated peptides to define the minimal linear epitopes recognized by 3 G10 and 2B4. Removal of amino acid residue <sup>34</sup>E at the amino terminus of the polypeptide <sub>34</sub>EWSGHDVTEIPNRRMF<sub>49</sub> showed reduced binding reactivity with MAb 3 G10 (OD<sub>492</sub> value dropping from 3.499 to 1.254). Similarly, deletion of the <sup>49</sup>F amino acid also exhibited reduced binding reactivity with MAb 3 G10 (OD<sub>492</sub> value dropping from 3.499 to 0.974). When both <sup>34</sup>E and <sup>49</sup>F were removed, MAb 3 G10 binding was completely abolished, indicating that <sub>34</sub>EWSGHDVTEIPNRRMF<sub>49</sub> is the minimal linear epitope required for optimal 3 G10 binding and <sup>34</sup>E and <sup>49</sup>F are important components of the peptide epitope (Fig. 5a).

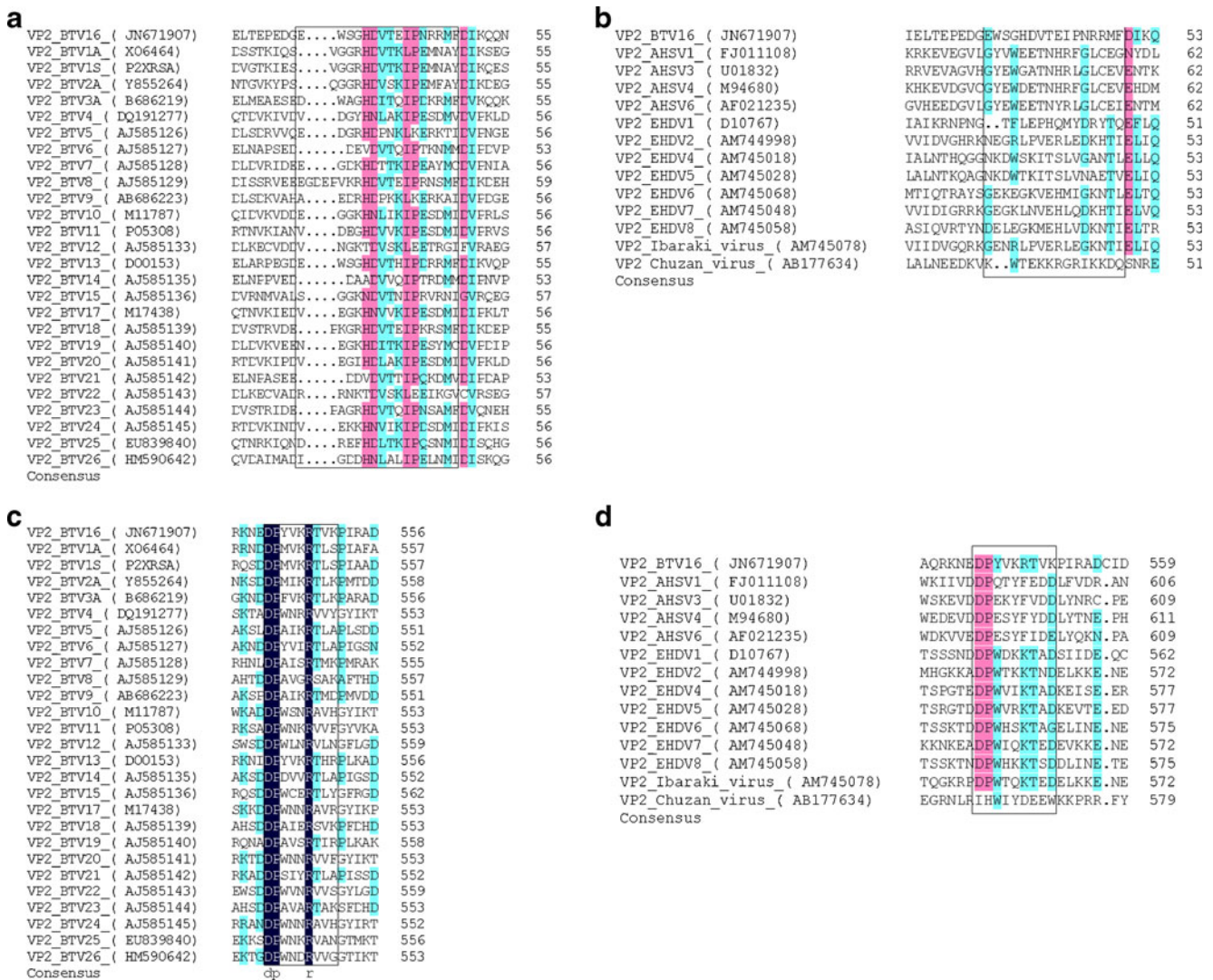
The initial peptide screen identified the polypeptide <sub>540</sub>KNEDPYVKRTVKPIRA<sub>555</sub> as the linear region

recognized by the MAb 2B4. A number of amino acids could be deleted from both the amino and carboxy termini of this polypeptide without significant reductions in 2B4 binding (Fig. 5b). All synthesized peptides containing the core <sub>543</sub>DPYVKRTVK<sub>551</sub> were recognized well by 2B4. Further truncation of the amino acid residue <sup>543</sup>D resulted in a complete loss of 2B4 binding, illustrating the importance of the amino acid residue <sup>543</sup>D in epitope maintenance



**Fig. 6** Reactivity of MAb 3 G10 and 2B4 with epitope regions of different BTV16 isolates which contain an amino acid substitution. **a** Peptides corresponding to the minimal MAb 3 G10 epitope region of a Japanese and Australian BTV16 isolate were synthesized and evaluated for reactivity with MAb 3 G10. **b** Peptides corresponding to the minimal MAb 2B4 epitope region of a Japanese and Australian BTV16 isolate were synthesized and evaluated for reactivity with MAb 2B4. The name and sequence of the peptides used are included below the figure. A VP7-specific MAb was used as a negative control. Error bars reflect the results of three experimental repeats





**Fig. 7** Amino acid alignments of the epitope regions from BTV serotype viruses and prototype orbiviruses. The sequences corresponding to the region encompassing the two BTV16 VP2 minimal linear epitopes were identified and aligned for a panel of BTV serotype viruses (including BTV1–26) and some prototype members of the genus *Orbivirus* in the family *Reoviridae*. **a, b** Sequence alignment of the region encompassing the MAb 3 G10 epitope  $_{34}EWSGHDVTEIPNRRMF_{49}$

for BTV1–26 (**a**) and prototype members of the genus *Orbivirus* in the family *Reoviridae* (**b**). **c, d** Sequence alignment of the region encompassing the MAb 2B4 epitope  $_{543}DPYVKRTVK_{555}$  for BTV1–26 (**c**) and prototype members of the genus *Orbivirus* in the family *Reoviridae* (**d**). The defined epitopes and their corresponding region in BTV1–26 and selected orbiviruses are boxed

(Fig. 5b). This work defined  $_{543}DPYVKRTVK_{551}$  as the minimal linear peptide recognized by 2B4.

Amino acid sequence alignments of a number of BTV16 strains isolated from different regions indicated that these two linear epitopes are highly conserved among BTV16 strains. The corresponding epitope regions of BTV16 strains containing a single amino acid substitution relative to the reference epitope sequence we identified were synthesized and tested for reactivity with the MAbs by indirect ELISA. Despite the amino acid substitution found within some BTV16 isolates, both BTV16 serotype-specific MAbs recognized the corresponding epitope regions of different BTV16 strains isolated from different regions (Fig. 6).

These two BTV16 VP2-specific MAbs and their corresponding binding epitopes can provide a foundation for the establishment of new serotype-specific differential diagnostic approaches.

Mapping epitopes using MAbs has become a powerful tool to study protein structure and has been used to diagnose diseases and design marker vaccines (Sukupolvi-Petty et al. 2010; Kouzmitcheva et al. 2001; Langeveld et al. 1994). In this study, we also used DNASTAR software (<http://www.dnastar.com/>) to analyze features of the BTV16 VP2 protein (GenBank accession number JN671907). This analysis suggested that the two linear epitopes identified in this study and their peripheral regions have a high degree of

hydrophilicity, a high antigenic index, and a positive surface probability (data not shown), suggesting that these epitopes stand a good chance of exposing on the surface of the VP2 tertiary structure. This analysis is consistent with our IFA results which showed that both MAbs 3 G10 and 2B4 specifically reacted with BTV16-infected cells (Fig. 2b). These results may provide some insight into the three-dimensional structural properties of the BTV VP2 protein.

In conclusion, we developed two BTV16 VP2-specific MAbs and defined the linear peptide epitopes within the VP2 protein recognized by each antibody. The two BTV16 VP2-specific MAbs and their defined linear peptide epitopes may be used in the establishment of serotype-specific differential diagnostic tools and vaccine development.

**Acknowledgments** This study was supported by the Special Fund for Agro-scientific Research in the Public Interest (20120356), Fundamental Research Funds for Central Public Welfare Research Institutes (ZGKJ201105), and Fundamental Research Funds for Central Public Welfare Research Institutes (0302012016).

**Conflict of interest** The authors declare that they have no conflict of interest.

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