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2 Identification of an Epitope within the Bovine Herpesvirus 1 Glycoprotein E Cytoplasmic
3 Tail and Use of a Monoclonal Antibody Directed Against the Epitope for the
4 Differentiation between Vaccinated and Infected Animals
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Abstract:

We constructed a recombinant bovine herpesvirus type 1 triple mutant virus (BoHV-1 tmv) that lacks UL49.5 residues 30-32 and 80-96, gE cytoplasmic tail (gE CT) residues 452-575 and the entire 435 bp long Us9 ORF. To develop a gE CT-specific blocking ELISA test that is necessary to distinguish the BoHV-1 tmv vaccinated calves from the wild-type (wt) virus-infected calves, a mouse monoclonal antibody (mAb) 2H8F3 was generated by using the E. coli expressed gE CT residues 452-575. Further, by performing a PEPSCAN analysis of 12 mer overlapping peptides spanning the entire gE CT, the epitope sequence recognized by the mAb2H8F3 was mapped within the gE CT residues ₄₉₉SDDDGPASN₅₀₇. A blocking ELISA test was then developed for detecting antibodies in wild-type BoHV-1 infected calves against the gE CT epitope specified by ₄₉₉SDDDGPASN₅₀₇. The assay is based on the use of HRP conjugated mAb2H8F3 and the E. coli expressed gE CT protein as an indicator antibody and a coating antigen, respectively. In this assay, serum from entire gE-deleted and BoHV-1 tmv-infected calves scored negative, whereas serum from calves infected with BoHV-1 wt scored positive. Therefore, the gE CT-ELISA, based on the mAb2H8F3 and E. coli expressed gE CT protein, is suitable for differentiating the wt virus-infected and BoHV-1 tmv-vaccinated cattle.

1. Introduction:

Bovine herpesvirus type 1 (BoHV-1) is an important viral pathogen of cattle that can cause severe respiratory tract infections known as infectious bovine rhinotracheitis (IBR), abortion in pregnant cows, and is an important component of bovine respiratory disease complex (BRDC) (Jones and Chowdhury, 2007; Jones and Chowdhury, 2010). The BRDC is a multifactorial disease in cattle which involves initial viral respiratory infection that may include BoHV-1, bovine respiratory syncytial virus (BRSV) and bovine viral diarrhea virus (BVDV) followed by secondary bacterial infection and severe bronchopneumonia. BRDC costs the US cattle industry alone more than \$ 1 billion annually (Jones and Chowdhury, 2007; Jones and Chowdhury, 2010). The ability of BoHV-1 to immunosuppress the infected cattle (Koppers-Lalic et al., 2005; Nataraj et al., 1997), to establish a lifelong latent infection in the trigeminal ganglia (TG) of infected animals, to reactivate from latency upon stress, and to be transported anterogradely from neuron cell bodies in the TG to axon termini in the nasal and upper respiratory epithelium followed by replication in the upper respiratory tract, predisposes the latently infected animals to BRDC (Jones and Chowdhury, 2007; Jones and Chowdhury, 2010). Therefore, BoHV-1 is considered to be an important initiator of the BRDC.

Currently, the only vaccine allowed in the EU countries against BoHV-1 is the entire gE-deleted BoHV-1 marker vaccine and by using the marker vaccine BoHV-1 has been eradicated in a number of European countries. Recently, we have determined that BoHV-1 triple mutant virus (tmv) was a safe vaccine as is the gE-deleted vaccine virus, but the protective vaccine efficacy of the BoHV-1 tmv against the BoHV-1 wt challenge was significantly better than the gE-deleted BoHV-1 (Chowdhury et al., 2014). The BoHV-1 tmv lacks; i) the UL49.5 residues 30-32 and 80-96 which down regulates MHC-I and causes transient immunosuppression in an infected animal (Wei et al., 2012; Wei, Wang, and Chowdhury, 2011), ii) the gE cytoplasmic tail (CT) residues 452-575 that are virulence determinant (Liu et al., 2008) and (iii) the entire 435 bp long

Us9 ORF which, along with the gE CT residues 452-575, together or individually, are essential for the BoHV-1 anterograde neuronal transport from the TG to nasal epithelium and nasal virus shedding after reactivation from latency (Brum et al., 2009; Butchi et al., 2007; Chowdhury et al., 2011; Chowdhury et al., 2010; Chowdhury et al., 2006)

The gE-deleted marker vaccine allows the differentiation of the wild type (wt) BoHV-1-infected from the vaccinated cattle (DIVA vaccine) which is important for the enforcement of the BoHV-1 eradication program. In the field, the gE DIVA vaccine is being used in conjunction with a serological marker test marketed by IDEXX. We have tested the suitability of the IDEXX ELISA test to distinguish the BoHV-1 tmv-vaccinated calves from the BoHV-1 wt-infected calves and the results (data not shown) showed that the IDEXX test was not suitable for distinguishing the BoHV-1 tmv-vaccinated calves from that of the wt virus-infected calves. This is because the BoHV-1tmv has retained the 2/3rd of the gE coding region (1-451 residues of gE ecto and transmembrane domains) and only the gE carboxy terminal 124 aa (also designated as the cytoplasmic tail of gE) are deleted. Therefore, a serological marker test that can distinguish the BoHV-1tmv vaccinated animals from the BoHV-1 wt infected animals is necessary before the BoHV-1 tmv based superior marker vaccine could be introduced in the field.

In this study, one of our objectives has been to develop a serological marker test that distinguishes the BoHV-1 tmv-vaccinated calves from the BoHV-1 wt virus-infected calves. The data presented shows that using bacterially expressed gE CT-specific protein and a horseradish peroxidase (HRP)- conjugated monoclonal antibody specific for an epitope within the gE CT, we developed a suitable competitive ELISA test that distinguished both the entire gE-deleted and gE CT-deleted (BoHV-1 tmv) from the wt virus-infected calves.

2. Materials and Methods.

2.1. Cells and virus strain. The MDBK cell line used for virus propagation was maintained as

described earlier. BoHV-1 tmv (containing UL49.5 residues 30-32 and 80-96, gE residues 452-575 and entire Us9 gene deletions) and BoHV-1 gE-deleted (entire gE ORF-deleted) viruses were constructed earlier (Brum et al., 2009; Wei et al., 2011). The BoHV-1 Cooper (Colorado-1) strain (ATCC Cat # CRL-1390, Manassas, VA) was used as the reference wild type strain.

2.2. Monoclonal/polyclonal antibodies.

Mouse anti His-tag mAb (Genscript Cat # A00186) and/or Goat anti gE CT-specific antibody (Chowdhury et al., 2014) goat anti mouse-HRP conjugated polyclonal antibody (Thermo Scientific Cat # 32430) were used to confirm reactivity and specificity of the recombinant protein and mAb respectively.

2.3. Construction of gE CT expressing pET-43.1a plasmid.

BoHV-1 gE CT nucleotide sequences coding for the residues 451-575 (GenBank accession# AJ004801) (Fig. 1A) were synthesized (Genscript), after codon optimization in favor of expression in *E. coli*, along with a Nde I restriction site followed by sequences coding for a methionine and 6 histidine residues on the left (5' end) and a stop codon followed by XhoI restriction site on the right (3' end) (Fig. 1B and C). The 411 bp long NdeI/XhoI fragment was then inserted into the NdeI-XhoI sites of pET-43.1a (Novagen) to create the plasmid clone pET-43.1a gE CT-His. The pET-43.1a gE CT-His construct was verified by sequencing. The predicted molecular weight of the *E. Coli* expressed purified gE CT-His (Fig. 1D) was verified by SDS-PAGE and by MALDE-TOF mass spec analysis (Genscript).

2.4. Production and characterization of gE CT-specific mouse monoclonal antibody (mAb) mAb2H8F3.

Protein purification, immunization of mice, production of hybridomas, and purification of the mAb 2H8F3 from the ascites fluid and conjugation of the mAb with HRP (horseradish peroxidase) were performed by Genscript.

2.5. Purification of gE CT-His from *E. coli*, immunoblotting analysis and generation of mAb.

Briefly, the E. coli expressed gE CT-His protein was obtained from inclusion bodies, solubilized with 8M Urea and purified in two steps by Ni column and Superdex 200 (GE Healthcare). The purified E Coli expressed gE CT-His protein was tested for reactivity with anti-His antibody (Genscript cat. # A00186). Mice were immunized with the purified gE CT-His protein and anti-His negative, gE CT-specific hybridomas were selected for further processing. Hybridomas producing gE CT-specific antibodies were selected for the production of mouse ascites.

2.6. Western blot analysis of the recombinant gE CT-His protein and gE CT-specific mAb.

The gE CT-His antigen protein samples were run on an SDS-PAGE (10% or 12%) and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in PBS and/or TBS for 1 hr at room temperature (RT), washed with .05% tween 20 in PBS (PBST) or TBS (TBST) and incubated with antibodies (0.5 µg/ml diluted in PBS or TBS for 2-3 hrs or overnight at RT). After washing, the membrane was incubated either with secondary antibody goat anti mouse HRP (Thermo Scientific Cat # 32430) or with the secondary antibody IRDye800CW goat anti-mouse IgG (LI-COR Biosciences) at RT for 1 hour. After washing, the membrane was visualized either with Pierce ECL western blotting substrate/autoradiography or under Odyssey scanner (LI-COR, Odyssey V3.0).

2.7. Synthesis of biotinylated overlapping peptides and epitope mapping ELISA.

Thirty nine 12 mer overlapping peptides (Table 1) spanning the entire 124 aa residues of gE CT (Fig. 1a) were synthesized (Table 1) and biotinylated (Genscript). The biotinylated peptides (20 µg/ml) were captured in 96-well microtiter plates pre-coated with 100 µl of 10 µg/ml streptavidin (NEB, Cat # A00160). In addition, a microtiter plate was coated with purified antigen protein gE CT-His (2 µg/ml) in 100 µl coating buffer at 4°C overnight. The plates were then blocked with the blocking buffer (5% skimmed milk in PBS) at 37°C for 2 hours. After washing, the plate with washing buffer (PBS with 0.05% tween), 100 µl of 1 µg/ml mAb 2H8F3 was added to the

plates and incubated at 37°C for 2 hours. The plates were washed with washing buffer (PBST) and incubated further with 100 µl of secondary antibody (0.1 µg/ml goat anti-mouse IgG [HRP]) at 37°C for 1 hour. After washing, the reaction was developed with 100 µl TMB substrate for 10 minutes at room temperature. The reaction was stopped by adding 100 µl of 1 M HCl. The absorbance of each well was measured at 450 nm using a spectrometer. An uncoated well was set as the blank control. The peptide library incubated with only the secondary antibody was set as the negative control.

2.8. Infection of calves. Animal infection, handling, sample collection and euthanasia protocols were previously approved by the LSU Institutional Animal Care and Use Committee. To determine the differential serological marker property of BoHV-1tmv in infected/vaccinated calves compared with the BoHV-1 gE-deleted and BoHV-1 wt-infected calves, twelve BoHV-1 and BVDV negative, 4-month-old cross-bred bull calves were selected and randomly assigned into 3 groups consisting of 3 calves (BoHV-1 wt), 4 calves (BoHV-1 gE-deleted) and 5 calves (BoHV-1 tmv). Calves in each group were housed at the LSU large animal isolation facility well isolated from each other in separate rooms containing 2-3 calves in each room. Calves in each group were infected intranasally with 1×10^7 PFUs of the respective virus/nostril (Total 2×10^7 PFUs/calf). Following infection, nasal swabs were collected every other day starting at 2 days post infection (dpi) to 12 dpi. Serum samples were collected at days 0, 14, and 28 dpi. aliquoted and stored in -80 C until further analysis.

2.9. Competitive ELISA tests. Competition ELISA was performed to detect the presence or absence of the gE CT-specific antibody among the three groups; wild type (WT), BoHV-1 tmv and gE deleted (gEΔ) virus infected calves serum samples. This was accomplished by collecting samples of WT, BoHV-1 tmv, and gEΔ calf sera at day 0 (prior to infection) 14 dpi and 28 dpi and testing them for the presence of the gE antibody.

ELISA plates (Costar, #3590) were coated overnight at 4°C with 200µl/well (approx. 100ng) of gE CT-His protein antigen in a coating buffer (30mM Na₂CO₃/70mM NaHCO₃ pH9.6). After washing with 0.05% Tween 20-PBS pH 7.4 (PBST) followed by 1xPBS pH 7.4 (PBS), the plates were blocked with 1% IgG free bovine serum albumin (BSA) in PBS at 37°C for 2 hours. After washing, the plates were incubated overnight, at 4°C, with 100µl of calf sera diluted 1:1 in 0.1% PBST. The next day, the plates were washed and incubated for 1 hour at room temperature with 100µl of HRP conjugated (Genscript) BoHV-1 gECT-specific mAb 2H8F3 (see below) diluted 1:10,000 in 0.05% PBST/well. Plates were washed and 150µl of TMB substrate was added to each well and incubated at room temperature in the dark for 15 minutes. 75µl of 1M H₂SO₄ was added to stop the reaction. Finally, optical density (OD) of each well was read in a microtitration plate reader (SpectraMax M2, Molecular Devices with Soft Max Pro 4.8) at 450nm.

2.10. Statistical Analysis: The SAS® (version 9.4, SAS Institute, Cary, NC) mixed procedure was used to analyze the competitive ELISA data with a repeated measures analysis of variance in a mixed effects model. Fixed effects included Sample, DPI and the interaction Sample*DPI. The random effect in the model was Calf (Sample). When overall differences were found, post hoc comparisons were made with pairwise “t” tests of least-squares means. All comparisons were considered significant at $p \leq 0.05$.

3. Results.

3.1. Immunoblotting analysis of recombinant BoHV-1 gE CT-His recombinant protein and assessment of the anti BoHV-1 gE CT-specific mAb2H8F3.

Molecular weight of the E. coli expressed recombinant gE CT-His protein is predicted to be 14.3 kD (Web.ExPASy.org) (Fig. 1D). Immunoblotting result depicted in Fig 2A shows that E. coli expressed gE CT-His protein reacted specifically to anti His antibody. However, molecular mass of the E. coli expressed gE CT-His protein was slightly higher than the predicted molecular mass 14.3 kD. Therefore, MALDI-TOF mass spec analysis was performed (Genscript) and based

on the intact mass, the molecular weight of the protein was very similar as the predicted molecular weight (Fig. 3). Subsequently, a BoHV-1 gE CT-specific mouse monoclonal antibody (mAb2H8F3) was generated by immunizing mice with the gE CT-His antigen protein (Genscript). Immunoblotting analysis results also show that the E. coli expressed gE CT-His (Fig. 2B) is recognized by mAb2H8F3 (Fig. 2C) but not by anti GST-specific antibody (Fig. 2D). Additionally, mAb2H8F3 reacted specifically with the BoHV-1 wt gE-specific 94 kD band expressed in BoHV-1 wt virus-infected cells but not with 68 kD band lacking the gE CT in BoHV-1 tmv-infected cells (Fig. 4A). These results demonstrated that mAb2H8F3 recognized an epitope within the 124 amino acids of gE CT.

3.2. Epitope mapping by overlapping peptide ELISA (PEPSCAN) of mAb2H8F3.

To map the epitope sequences specified by the BoHV-1 gE CT-specific mAb2H8F3, thirty nine 12 mer overlapping peptides (Table 1) spanning the entire 124 aa residues (Fig. 1a) were synthesized and analyzed for binding with the gE CT-specific mAb2H8F3. The ELISA test results presented in Tables 2A and Fig. 5 show that the mAb 2H8F3 reacted specifically with the two overlapping peptides #16 (⁴⁹⁶DDSDDDGPASN₅₀₇) and # 17 (⁴⁹⁹SDDDGPASNPPA₅₁₀). Therefore, the epitope sequences of the mAb2H8F3 maps within gE residues shared by the two overlapping peptides # 16 and #17 (Tables 2 &3). Analysis of the two peptide sequences shown in Table 3 clearly demonstrate that the 9 amino acids “⁴⁹⁹SDDDGPASN₅₀₇” are common in the two overlapping peptides. Therefore, we conclude that the epitope of mAb2H8F3 is specified by 9 gE CT residues which are gE residues ⁴⁹⁹SDDDGPASN₅₀₇ (Table 3).

3.3. Virus isolation, plaque assay and virus neutralization assays.

Virus titrations were performed as described earlier (Chowdhury, 1996). BoHV-1 specific virus neutralization (VN) titers of the calf sera were determined as described earlier (Butchi et al., 2007). The VN titers were expressed as the reciprocal of the highest dilution of a serum that

caused a 50 % reduction of the standard number of plaques used in the test relative to the virus control (without serum).

3.4. Assessment of the serological marker assay to distinguish BoHV-1 WT-infected calves from the BoHV-1 tmv and/or gE-deleted virus-infected calves.

The direct ELISA test was optimized using a series of gE CT coating antigen concentrations and HRP conjugated mAb2H8F3 dilutions. Based on the analyses (data not shown), the optimal amount of gE CT-His coating antigen and HRP-conjugated mAb 2H8F3 (indicator marker antibody) were selected to be 100 ng of gE CT-His antigen and 1:10,000 dilution of the HRP conjugated mAb, respectively. For the blocking ELISA test, inactivated calf serum samples obtained prior to intranasal inoculation (0 dpi), at 14 dpi and at 28 dpi were incubated with the gE CT test antigen to determine their subsequent competitive inhibition/blocking of HRP conjugated indicator marker antibody binding. Inactivated fetal calf serum (available commercially, Atlas Biologicals), and hyper immune BoHV-1 wt-specific calf serum produced by multiple buster I/M immunizations using purified BoHV-1 as antigen were used as negative and positive controls, respectively. Measured OD's of each serum sample at 450nm was used to calculate either S/N ratio or percent inhibition as follows:

$$\text{Percent blocking} = \left(\frac{A_{450} \text{ OD negative control} - A_{450} \text{ OD sample}}{A_{450} \text{ OD negative control}} \right) \times 100$$

$$\text{S/N ratio} = \left(\frac{A_{450} \text{ OD sample}}{A_{450} \text{ OD negative control}} \right)$$

If the S/N ratio is less than or equal to 0.6, the sample is classified as positive for the presence of gE CT-specific antibody. If the S/N ratio is greater than 0.7, the sample is classified as negative for antibodies to the gE CT antigen. If the SN ratio is between 0.6-0.7, then the sample is classified as a suspect. To determine the cutoff value of the gE CT ELISA, for positive blocking of the wt virus infected test calf serum at 28 dpi, first the mean blocking percentage and the standard

deviation of the 12 calf sera before virus inoculation (at day 0) (Table 4) was calculated. The mean blocking percentage and the standard deviation of these calves at day 0 was 10.47 and 6.19, respectively, and the mean blocking percentage plus 3 times the standard deviation was 29.04%. Therefore, the cutoff value of the BHV-1 gE CT ELISA was set at 30%.

In the competitive/blocking ELISA tests, serum samples of all the 12 calves collected prior to infection (0 day) were negative for gE CT epitope-specific antibody (SN ratio > 0.7 and blocking percent less than 30%). Two of the three BoHV-1 wt infected calf serum samples collected at 28 dpi tested positive (S/N ratio < 0.6 and blocking percent > 30%) while one wt-infected calf serum tested in the suspect category at 28 dpi (S/N ratio 0.7 and blocking percent 29.66%) (Table 4). The ELISA test results also showed that 3 out of 4 calves infected with a gE-deleted virus tested negative at 28 dpi while one had a slightly lower S/N ratio (.685) and 31.48% blocking which was in the suspect category. Nevertheless, all 5 calves infected with BoHV-1 tmv tested negative in the competitive ELISA both at 0 and 28 dpi. When the serum samples collected from the same animals at 14 dpi were tested in the blocking ELISA, the S/N ratio and blocking percentage of all the calves sera including the 3 wt-infected calves were in the negative category (>.7 and less than 30%, respectively) and the values for the wt-infected calves were not noticeably different from the BoHV-1 gE Δ and BoHV-1 tmv infected calves (data not shown).

Statistical analysis revealed that there was an overall significant interaction for Sample*DPI ($p=0.021$) with respect to S/N Ratio. Post hoc comparisons indicated that there were significant differences on 28 dpi between the BoHV-1 wt-infected versus BoHV-1 gE ($p=0.0006$) and BoHV-1 tmv ($p=0.0025$) viruses-infected calves.

To determine whether the blocking ELISA results have some correlation with the serum VN titers of the calves, we compared the S/N ratio of the serum samples (Table 4) with the corresponding VN titers (Table 5). The analysis showed that there was no correlation between the S/N ratio of the serum samples and their corresponding VN titers.

4. Discussion.

We initiated these experiments to develop a serological marker test that can distinguish the recently described triple gene mutated vaccine strain BoHV-1 *tmv*- vaccinated calves from the BoHV-1 wt virus-infected calves. First, BoHV-1 gE residues spanning the entire cytoplasmic tail (124 aa) were expressed in *E. coli* and the purified gE CT protein was used to generate BoHV-1 gE CT-specific mAb. Second, the epitope sequence specific for the mAb was determined. Third, using the purified bacterially expressed gE CT proteins and HRP conjugated gE CT epitope-specific mAb a blocking ELISA test was developed to test DIVA characteristics of BoHV-1 gE-deleted- and BoHV-1 *tmv*-vaccinated calf serum compared with several wt-infected calf serum. The results demonstrated that the gE CT based DIVA ELISA test can distinguish the gE-deleted- and BoHV-1 *tmv*- vaccinated calves from the wt-infected calves.

Even though the current entire gE-deleted marker vaccine has issues with respect to the sensitivity of the marker gE-ELISA assay, many European countries have decided to start an eradication program based on the entire gE-deleted vaccine virus and on the use of accompanying gE-ELISA kit (Anonymous, 1996). As noted above, the IDEXX gE ELISA kit was not suitable for distinguishing BoHV-1 *tmv* infected calves from that of wt-infected calves. This could be due to the fact that in BoHV-1 *tmv*, the entire gE ectodomain is intact and only the gE carboxy terminal 123 aa (also designated as the cytoplasmic tail of gE) deleted and because the IDEXX ELISA kit uses the HRP conjugated mAb 67 that recognizes a conformational epitope formed by the gE/gI complex and which is located within the gE ectodomain (Rijsewijk et al., 2000; Tyborowska et al., 2000; Van Oirschot et al., 1997; Wellenberg et al., 1998).

In our gE CT-specific serological marker ELISA assay, we used the HRP conjugated mAb 2H8F3 that recognized the linear epitope sequence ₄₉₉SDDDGPASN₅₀₇ located within the gE cytoplasmic tail. Most of the gE epitopes reported so far and used in the IDEXX ELISA kit are localized within the gE ectodomain(Rijsewijk et al., 2000; Tyborowska et al., 2000; Van Oirschot

et al., 1997; Wellenberg et al., 1998). Therefore, the epitope we have identified is the only one known so far to be within the gE CT. Analysis of predicted gE amino acid residues revealed that the gE cytoplasmic tail sequences have significantly more antigenic and hydrophilic domains (Fig. 6) when compared with the gE ectodomain (data not shown) and that the residues ⁴⁹⁹SDDDGPASN₅₀₇ are within the gE CT domain predicted to be highly antigenic and hydrophilic. These epitope specific residues are also conserved in the European BoHV-1.1 strain Lam and in BoHV-1.2 strain ST (Leung-Tack, Audonnet, and Riviere, 1994). Our competitive ELISA test results (Table 4) also showed that while the percent blocking and S/N ratio values of hyperimmunized calf serum against BoHV-1 were 71% and .29, respectively, the corresponding values in two of the three calves infected intranasally once with 2×10^7 PFUs of BoHV-1 wt, at 28 dpi, were 40% (> 30%) and .55 (< .6) , respectively. These results, suggested that both the hyperimmunized calf and experimentally infected calves sera reacted specifically with the epitope specified by the gE CT residues ⁴⁹⁹SDDDGPASN₅₀₇ contained within the gE CT protein sequence and blocked or competed for the binding of the HRP conjugated mAb 2H8F3 specific for the epitope sequence. Importantly, a significantly increased percentage of blocking (71%) and lower S/N ratio (.290) in the case of hyperimmune calf serum compared with the calves receiving one intranasal inoculation indicated that the hyperimmunized calf generated an increased amount of the gE CT epitope-specific antibody because it received multiple I/M injections of purified wt virion samples. While the sera of calves, inoculated intranasally with BHV-1 wt, at 28 dpi had more than the cutoff value (> 30% blocking) for percent blocking and lower S/N ratio (.6), the sera of the same calves at 14 dpi did not achieve the cutoff value of 30% or more percent blocking of the reactivity of the HRP conjugated indicator marker mAb and less than .6 S/N ratio (data not shown). These results also indicated that our ELISA test is sensitive enough to detect the antibody against the gE CT epitope in the wild type-infected calves as early as 28 dpi but not at 14 dpi. Similar sensitivity for the IDEXX ELISA kit has also been reported earlier(de Wit et al., 1998). In the case of BoHV-1 tmv-infected calves all five calves showed less than 15% inhibition and their S/N ratios

1 ranged from .850-.913. Taken together, our gE CT-specific serological marker assay could
2 distinguish between the wt- and the BoHV-1 tmv-infected calves as early as 28 dpi. In this study,
3 we did not determine how soon after the initial intranasal inoculation, the wt-infected and BoHV-
4 1tmv vaccinated calf sera are distinguishable in the marker ELISA test; this needs to be performed
5 in a future study. While all five of the BoHV-1 tmv vaccinated calf sera tested negative in the
6 marker ELISA test, one (calf # 2559) out of 3 wt-infected calves and one (calf # 27) out of 4 gE-
7 deleted virus infected/vaccinated calves tested as suspects (Table 4) because their S/N ratios
8 were .7 (calf # 2559) and .68 (calf # 27), and the corresponding percent blocking values were
9 29.66 (calf # 2559) and 31.48 (calf # 26). Serum neutralization titers against the BoHV-1 wt for
10 the calf # 2559 (wt) and # 27 at 28 dpi were 48 and 23.7, respectively (Table 5). Therefore, the
11 S/N ratios and percent blocking values in the ELISA tests did not correspond to their serum
12 neutralization titers. Similar findings have been reported in earlier studies (Van Oirschot et al.,
13 1997). Since these animals were challenged with the BoHV-1 wt virus on that day after the serum
14 samples were collected, we could not test their serum in our ELISA at a later time point. Future
15 studies will further validate our serological marker tests under field conditions by using more
16 calves in each group and the sera collected over a longer period (3-4 months' time).

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Figure legends.

Fig. 1. Design and expression strategy of recombinant gE CT protein based upon predicted BoHV-1 gE CT amino acid sequences. **A.** Predicted amino acid sequences of BoHV-1 gE CT (BoHV-1 gE residues 451-575). **B.** BoHV-1 gE CT coding nucleotide sequences codon optimized for *E. coli* (Genscript). **C.** BoHV-1 gE CT coding sequences, cloned into the recombinant plasmid pET-43.1a gE CT-His at the NdeI/XhoI restriction sites, NdeI restriction site and poly His coding sequences were fused in frame upstream of the gE CT coding sequence for the residue 451 and two stop codons plus an XhoI restriction site were added immediately downstream of the gE CT carboxy terminal coding sequence for the residue 475 **D.** Estimation of isoelectric point (pI) and molecular weight (MW) of the recombinant BoHV-1 gE CT-His.

Fig. 2. SDS-PAGE/immunoblotting analyses of recombinant BoHV-1 gE CT-His (gE-CT His) and anti BoHV-1 gE CT-specific mAb 2H8F3. A. Coomassie blue stained 12% SDS-PAGE containing purified gE CT –His (lane 1) and immunoblotting results of the same gE CT-His- specific band with Anti His Ab (lane 2). B. Coomassie blue stained 12% SDS-PAGE containing gE CT-His protein under reduced (lane 1) and non-reduced (lane 2) conditions. Immunoblotting results of the samples in B with BoHV-1 gE CT-specific mAb 2H8F3 (panel C) and with anti-GST mAb (panel D).

Fig. 3. MALDI-TOF Mass Spec plot of gE CT-His protein.

Fig. 4. Immunoblotting analysis of mAb 2H8F3 against BoHV-1 wt and BoHV-1 tmv-infected cell lysates. A. Immunoblotting analysis of mock-, BoHV-1 wt- and BoHV-1 tmv-infected MDBK cell lysates separated in a 10% SDS-PAGE with gE CT-specific mAb 2H8F3. B. Immunoblotting analysis of an identical Western blot with a gE ectodomain-specific rabbit polyclonal antibody (Chowdhury et al., 2014). Note that approx. 68 kD gE Δ CT-specific band not recognized by the mAb 2H8F3 in panel A is recognized by the gE ectodomain-specific rabbit polyclonal antibody in panel B.

Fig. 5. Graphic presentation of epitope mapping ELISA results. No.1-39 are the binding of the peptides (sequences shown in table 1) to the test antibody mAb 2H8F3; No.40 is the binding of mAb 2H8F3 to streptavidin, which is set as blank control; and No. 41 is the binding of the mAb 2H8F3 to purified E. coli expressed recombinant gE CT-His protein which was used for the production of the mAb.

Fig. 6. Analysis of predicted gE CT residues 450-575 for antigenicity, hydrophilicity, surface probability, and secondary structures using the program DNASTAR Protean 3D. Hydrophilicity plot was calculated using the method after Kyte-Doolittle. Alpha plot, Beta plot and Turn plot were calculated using the method of Chou-Fasman. Surface probability was calculated using the method of Emini. The method of Jameson-Wolf was used to calculate antigenicity. The gE CT

- 1 residues, $_{499}\text{SDDDGPA}_{507}\text{SN}$ recognized by the mAb 2H8F3 are shown between the two vertical
- 2 lines.

1	ASQKRTYDILNP	14	DEDSFADDDSD	27	PANGTRSSRSGF
2	KRTYDILNPFPG	15	SFADDDSDDDGP	28	GTRSSRSGFKVW
3	YDILNPFPGPVYT	16	DDSDDDGPN	29	SSRSGFKVWFRD
4	LNPFPGPVYTSLP	17	SDDGPNPPA	30	SGFKVWFRDPLE
5	FGPVYTSLPNE	18	DGPNPPADAY	31	KVWFRDPLEDDA
6	VYTSLPNEPLD	19	ASNPPADAYDLA	32	FRDPLEDDAAPA
7	SLPNEPLDVVV	20	PPADAYDLAGAP	33	PLEDDAAPARTP
8	TNEPLDVVVPVS	21	DAYDLAGAPEPT	34	DDAAPARTPAAP
9	PLDVVVPVSDDE	22	DLAGAPEPTSGF	35	APARTPAAPDYT
10	VVVPVSDDEFSL	23	GAGEPTSGFARA	36	RTPAAPDYTVVA
11	PVSDDEFSLDED	24	EPTSGFARAPAN	37	AAPDYTVVAARL
12	DDEFSLDEDSFA	25	SGFARAPANGTR	38	DYTVVAARLKSI
13	FSLDEDSFADDD	26	ARAPANGTRSSR	39	TVVAARLKSILR

Table 1. Overlapping 12 mer peptide library spanning the entire BoHV-1 gE CT (gE residues 452-575; GenBank accession # AJ004801).

Table 2a. Indirect ELISA of interaction between peptides and target antibody†

¹ 0.152	² 0.096	³ 0.093	⁴ 0.097	⁵ 0.089	⁶ 0.097	⁷ 0.099	⁸ 0.103	⁹ 0.083	¹⁰ 0.101	¹¹ 0.094	¹² 0.095
¹³ 0.082	¹⁴ 0.095	¹⁵ 0.087	¹⁶ <u>0.450</u>	¹⁷ <u>0.655</u>	¹⁸ 0.082	¹⁹ 0.087	²⁰ 0.102	²¹ 0.098	²² 0.086	²³ 0.101	²⁴ 0.098
²⁵ 0.105	²⁶ 0.097	²⁷ 0.121	²⁸ 0.100	²⁹ 0.098	³⁰ 0.087	³¹ 0.081	³² 0.089	³³ 0.066	³⁴ 0.097	³⁵ 0.089	³⁶ 0.111
³⁷ 0.094	³⁸ 0.099	³⁹ 0.096	⁴⁰ 0.051	⁴¹ 2.359							

Table 2b. Indirect ELISA of interaction between peptides and secondary antibody†

¹ 0.062	² 0.074	³ 0.058	⁴ 0.058	⁵ 0.056	⁶ 0.063	⁷ 0.060	⁸ 0.059	⁹ 0.058	¹⁰ 0.061	¹¹ 0.059	¹² 0.063
¹³ 0.058	¹⁴ 0.060	¹⁵ 0.052	¹⁶ 0.056	¹⁷ 0.053	¹⁸ 0.053	¹⁹ 0.052	²⁰ 0.054	²¹ 0.053	²² 0.051	²³ 0.055	²⁴ 0.059
²⁵ 0.060	²⁶ 0.055	²⁷ 0.053	²⁸ 0.054	²⁹ 0.056	³⁰ 0.051	³¹ 0.052	³² 0.056	³³ 0.056	³⁴ 0.052	³⁵ 0.055	³⁶ 0.066
³⁷ 0.058	³⁸ 0.054	³⁹ 0.053	⁴⁰ 0.055	⁴¹ 0.057							

Table 2. ELISA results of epitope mapping for mAb 2H8F3.

†: The superscripts represented the peptide numbers (1-39). No.1-39 are the binding of the peptides to the mAb 2H8F3 as indicated by O.D measured at 450 nm; No.40, binding of mAb 2H8F3 to streptavidin was set as blank control; and No. 41, binding of the mAb 2H8F3 to purified E. coli expressed recombinant gE CT-His protein.

Peptide No.	Amino acid
16	⁴⁹⁶ DDD <i>SDDDGPA</i> <i>SN</i> ₅₀₇
17	--- ₄₉₉ <i>SDDDGPA</i> <i>SN</i> PPA ₅₁₀

Table 3. Deduced overlapping epitope sequences (bolded and italicized) contained within the peptide # 16 (gE residues 496-507) and peptide # 17 (gE residues 499-510). Note that gE residues 499-507 are shared in both the peptides which constitute the epitope in the gE CT recognized by the mAb2H8F3.

Groups	Samples	% Blocking		S/N Ratio		Positive?
Neg. Control	FBS	0		1		N
Pos. Control	BoHV-1-HI*	71.02		0.290		Y
	Calf #	0 dpi	28 dpi	0 dpi	28 dpi	
BoHV-1 WT	2309	10.29	44.88	0.897	0.551	Y
	2349	19.21	41.29	0.808	0.587	Y
	2559	19.66	29.66	0.803	0.703	Suspect
BoHV-1 gEΔ	26	15.84	27.30	0.842	0.727	N
	27	13.85	31.48	0.862	0.685	Suspect
	28	6.49	18.99	0.935	0.810	N
	32	1.47	15.49	0.985	0.845	N
BoHV-1 tmv	31	3.93	8.72	0.961	0.913	N
	33	9.35	10.16	0.906	0.898	N
	369	2.00	14.97	0.980	0.850	N
	379	13.40	15.40	0.866	0.846	N
	385	10.16	10.96	0.898	0.890	N

Table 4. Assessed competitive/blocking ELISA results of calf serum samples prior to infection (0 day) or at 28 days post infection (28 day) with BoHV-1 wt, BoHV-1 gEΔ and BoHV-1 tmv. S/N ratio and % inhibition values calculated from mean O.D of 3 replicate ELISA tests for each serum samples are shown. Note that our negative control, commercially available FBS (fetal calf serum) has 0% inhibition and the S/N ratio is 1 whereas the positive control hyperimmunized calf serum against BoHV-1 wt has 71% inhibition and 0.29 S/N ratio. If the S/N ratio is less than or equal to 0.6, the sample is classified as positive for the presence of gE CT-specific antibody. If the S/N ratio is greater than 0.7, the sample is classified as negative for antibodies to the gE CT antigen. If the S/N ratio is between 0.6-0.7, then the sample is classified as a suspect.

* Hyperimmunized (HI) calf serum against BoHV-1: The calf was immunized with purified BoHV-1 virion suspension by I/M multiple times.

Groups	Samples	VN titers*		
		0 dpi	14 dpi	28 dpi
BoHV-1 WT	2309	<2	60	64
	2349	<2	30	48
	2559	<2	40	48
BoHV-1 gEΔ	26	<2	14	28.9
	27	<2	13.8	23.7
	28	<2	12.7	18.2
	32	<2	14	29.0
BoHV-1 tmv	31	<2	17.9	23.7
	33	<2	12	20.8
	369	<2	7.9	10.5
	379	<2	13.5	17.7
	385	<2	12.6	17.8

Table 5: Serum VN titers of the calves infected with BoHV-1 wt, BoHV-1 gEΔ and BoHV-1 *tmv*.

* 50% serum neutralization (SN) or plaque reduction titers against BHV-1 wt (100 PFU's) are shown.

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1 ASQKRTYDIL NPFQPVYTSI PTNEPLDVVV PVSDEFSLD EDSFADDDSD DDGPASNPPA
61 DAYDLAGAPE PTSQFARAPA NGTRSSRSQF KVFWRDPLED DAAPARTPAA PDYTVVAARL
121 KSILR

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A

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1 GCATCGCAAA AGCGTACCTA TGATATTCTG AACCCGTTTG GTCCGGTCTA CACGAGCCTG
61 CCGACGAACG AACCGCTGGA TGTTGTTGTG CCTGTTAGTG ATGACGAATT TTCCTGGAT
121 GAAGACTCAT TCGCCGATGA CGATTCCGAC GATGACGGTC CGGCAAGCAA CCCGCCGGCA
181 GATGCTTATG ATCTGGCAGG TGCACCGGAA CCGACCTCTG GTTTTGCACG TGCTCCGGCG
241 AATGGCACGC GTAGCTCTCG CTCCGGTTTT AAAGTCTGGT TCCGCGATCC GCTGGAAGAT
301 GACGCGGCCC CGGCGCGTAC CCCGGCGGCA CCGGACTACA CCGTGGTTGC GGCCCGTCTG
361 AAGAGCATCC TCGCT

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B

Poly His-Tag

NdeI └──────────┘

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1 CATATGCATC ACCACCATCA CCACGCATCG CAAAAGCGTA CCTATGATAT TCTGAACCCG
61 TTTGGTCCGG TCTACACGAG CCTGCCGACG AACGAACCGC TGGATGTTGT TGTGCCTGTT
121 AGTGATGACG AATTTTCCCT GGATGAAGAC TCATTGCGCG ATGACGATTC GGACGATGAC
181 GGTCCGGCAA GCAACCCGCC GGCAGATGCT TATGATCTGG CAGGTGCACC GGAACCGACC
241 TCTGGTTTTG CACGTGCTCC GCGCAATGGC ACGCGTAGCT CTCGCTCCGG TTTTAAAGTC
301 TGGTTCCGCG ATCCGCTGGA AGATGACGCG GCCCCGGCGC GTACCCCGGC GGCACCGGAC
361 TACACCGTGG TTGCGGCCCC TCTGAAGAGC ATCCTGCGTT AATGACTCGA G

```

Stop Stop **XhoI**

C

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      10      20      30      40      50      60
MHHHHHHASQ KRTYDILNPF GPVYTSIPTN EPLDVVPVS DDEFSLDEDS FADDDSDDDG

      70      80      90     100     110     120
PASNPPADAY DLAGAPEPTS GFARAPANGT RSSRSGFKVW FRDPLEDDAA PARTPAAPDY

      130
TVVAARLKSI LR

```

Theoretical pI/Mw: 4.59 / 14314.50

D

Fig. 1

Fig. 2

Spectrum from 290011_C4_MS_02.wiff (sample 1) - 290011_50%ACN 1%FA_0.05mg/ml, +TOF MS (300 - 2000) from 3.019 to 3.139 min

Reconstruction, Input m/z: 1285.2 to 1798.1 Da, Input spectrum isotope resolution: Very Low (1000)

Fig. 3.

Fig. 4

Epitope Mapping ELISA

Fig. 5

gE CT (aa 450-575)

Fig 6.