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**PRODUCTION OF B VIRUS GLYCOPROTEIN D AND EVALUATION OF ITS
DIAGNOSTIC POTENTIAL**

by

CHADI FILFILI

Under the Direction of Dr Julia Hilliard

ABSTRACT

B virus diagnosis presents a challenge largely complicated by the asymptomatic infection of rhesus macaques, and extremely pathogenic fatal infections in humans. Humoral detection of antibodies is generally performed using whole virus antigen for which preparation requires strict biosafety measures and specialized BSL-4 facilities. As an alternative to utilizing B virus antigen, we describe the production of a truncated form of B virus envelope glycoprotein D, gD 287, in a baculovirus expression system, and evaluate its diagnostic potential as an antigen in recombinant ELISA. After purification and characterization, gD 287 was tested using 22 negative and 72 positive macaque sera samples previously classified using the traditional method. We find that sensitivity and specificity of the recombinant ELISA are dependent on antibody titer of tested serum and gD 287 shows good to excellent predictive potential for identification of positive sera with titers higher than 500.

INDEX WORDS: Serodiagnosis, Herpes virus, Glycoprotein D, Recombinant, ELISA, Rhesus macaque, cercopithecine.

**PRODUCTION OF B VIRUS GLYCOPROTEIN D AND EVALUATION OF ITS
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CHADI FILFILI

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

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Department of Biology

in the College of Arts and Sciences

Georgia State University

2008

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Chadi Nabih Filfili
2008**

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DIAGNOSTIC POTENTIAL**

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College of Arts and Sciences
Georgia State University
May 2008**

DEDICATION

To my parents Nabih and Najat whose perseverance is to me a permanent inspiration,
and to Tina, Andrew and Angelo for the joy and support they continue to be for me.

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I am ever indebted to Dr Julia Hilliard for giving me this wonderful opportunity by supporting my work and overall training and for her constant priceless guidance. My gratitude goes out to all those in Dr Hilliard's lab who contributed to my professional and personal development, especially Dr Ludmilla Pereygina for her continuous guidance throughout the project and Dr Irina Patrusheva for day-to-day aid with technical methods.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
B virus	Cercopithecine Herpesvirus 1
BHV-1	Bovine Herpesvirus 1
BSA	Bovine serum albumin
BSL-4	Biosafety Level 4
CHO	Chinese hamster ovary
CNS	Central nervous system
Cos7	African Green Monkey SV40-transfected kidney fibroblast cell line
CV	Column volume
ECL	Enhanced Chemiluminescence
ELISA	Enzyme linked immunosorbent assay
FPLC	Fast Performance Liquid Chromatography
gD	Glycoprotein D
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
hpi	Hours post infection

HSV-1	Herpes Simplex Virus 1
HSV-2	Herpes Simplex Virus 2
HveA	Herpesvirus entry receptor A
HVEM	Herpesvirus Entry Mediator
IFN- α	Interferon alpha
IPTG	Isopropyl β -D-1 thiogalactopyranoside
JEV	Japanese Encephalitis Virus
LB	Luria Broth
MOI	Multiplicity of infection
OD	Optical Density
ORF	Open Reading Frame
P/N	Positive/Negative ratio
PCR	Polymerase Chain Reaction
SA8	Simian Agent 8
SD	Standard Deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFM	Serum free medium
TE buffer	10 mM Tris, 1 mM EDTA, pH 7.5

I. INTRODUCTION:

The discovery of B virus marked the identification of the first simian host-associated virus. The pathogenesis of B virus in humans was first revealed to the scientific community in 1932 in a case study of researcher Dr W.B., who died from encephalitis following a bite from a rhesus monkey (Gay and Holden 1933; Weigler 1992). Extensive study of this case using virological and immunological methods resulted in the isolation of B virus. Described as antigenically related to herpes simplex virus (HSV), B virus experimentally injected into rabbits produced paralytic disease and death (Gay and Holden 1933; Sabin A B 1934). With the exception of intracerebral injection of B virus, experimental infection of rhesus macaques with this agent by various routes failed to produce the neurological disease. Moreover, neutralizing antibodies could be detected in the sera of these macaques during the convalescent phase of infection. All these observations were suggestive evidence that B virus is endemic to rhesus monkeys. Consistent with a general trend of alphaherpesviruses, the virulence and pathogenesis of B virus were manifested mainly in cross-species infections of other mammals, particularly New World primates, and humans (Palmer 1987; Weigler 1992). The wide use of rhesus macaques in research involving poliovirus and simian retroviral agents was accompanied by increasing incidents of B virus transmission to researchers and lab workers. B virus research historically included human case studies, serological surveys, experimental laboratory animal studies, and establishment of specific pathogen free colonies (Ward and Hilliard 1994; Hilliard and Ward 1999). A member of the subfamily *Alphaherpesvirinae* and genus *Simplexvirus*, B virus is a double-stranded DNA virus with a genome that consists of 156, 789 bp (Perelygina, Zhu et al. 2003). B virus pathogenesis

is challenging to study due to the risks associated with working with the virus and the costly biocontainment facilities necessary for handling the virus and experimentally infected animals.

The isolation of B virus in the mid 1930's opened the way for serological studies and identification of antibodies to B virus. Serological characterization of primary herpetic infections was well described by Buddingh et al in 1953. In 1954, Melnick et al isolated B virus from a rhesus monkey and showed that immune and convalescent B virus sera neutralized the isolate, confirming that antibodies to the isolate were present in monkey serum (Gay and Holden 1933; Sabin A B 1934; Melnick and Banker 1954).

The serological diagnosis of B virus progressed along with technological advances in antibody detection. A virus and an antibody had been shown to unite *in vitro* as early as 1927 by complement-fixation assays (Bedson 1929). In 1932, the time around which B virus was identified, the primary route of virus growth for research purposes was shifting from laboratory animals to chick embryos, a technique that would stay in use well into the 1960's. The development of cell culture techniques allowed virus growth *in vitro* and the subsequent identification of isolated viruses through the observation of virus-specific cytopathic effects on cultured cells. The ability to obtain high titer virus in cell culture was a necessary bridge to diagnostic assays and to the production of antibodies to viral antigens using experimental infections of laboratory animals. The development of monolayer cultures of kidney epithelial cells enabled the growth of B virus *in vitro*. The cytopathic effects of B virus were studied in greater detail using electron microscopy (Youngner 1954; Reissig and Melnick 1955). In 1956, J.S Youngner developed a plaque assay based system for testing B virus serum neutralization (Youngner 1956). Nevertheless, serodiagnosis of B virus was hindered by difficulties arising from viral infections with closely related viruses, i.e., HSV-1 and HSV-2 (Van Hoosier GL Jr 1961; Cabasso, Chappell et al. 1967; Aeda Y 1968).

With the advent of recombinant protein technology in the late 1970's, an interest developed in the use of recombinant analogs as antigens in diagnostic assays. The first reported laboratory production of a recombinant protein came in 1977 when somatostatin was expressed in *E coli* using recombinant DNA technology developed from Paul Berg's innovation in 1972 of methods to digest and join DNA molecules from different organisms (Jackson, Symons et al. 1972; Itakura, Hirose et al. 1977). Immunological assays using recombinant antigen became the method of choice where whole virus assays involved health hazards to the preparer, high cost, or low virus antigen yield. Recombinant antigen preparations were often found to have lower batch-to-batch variation than whole virus preparations, a property that promoted use of these as diagnostic reagents that enhanced diagnostic assay standardization. Although the authenticity of recombinant agents was often debated with regard to similarity with virus, some were shown to have higher specificity and exhibited less cross-reactivity than their whole virus counterparts. Total viral protein preparation often involves denaturation under harsh chemical treatment. The effects of such treatment on the integrity of discontinuous epitopes of viral proteins may result in failure to interact with antibodies induced by the virus during infection. In contrast, many recombinant proteins can be expressed and purified under more native conditions, resulting in forms closer to those encountered by the immune system during infection. Consequently, many laboratory-cloned proteins are diagnostically efficient antigens. For instance, Ebola recombinant nucleoprotein diagnostic assays produce a lower rate of false positives than those using complex antigen preparations from whole virus (Feldmann, Nichol et al. 1994; Simmons,

Porter et al. 1998; Groen, van den Hoogen et al. 2003). In HIV diagnosis, a number of immunoassays were developed using recombinant forms of *gag*, *env* and *pol*, the major immunodominant epitopes of HIV; these proved to be very valuable tools in the diagnosis of HIV infection, leading to safer blood transfusions since 1985 (Schultz, Aschauer et al. 1986; Steimer, Higgins et al. 1986; Dawson, Heller et al. 1988). A μ -capture ELISA employing recombinant nucleocapsid proteins of Hantavirus, Hantaan 76-118 and CG 18-20, as diagnostic antigens of hemorrhagic fever with renal syndrome resulted in assay sensitivity and specificity of 100% each (Zoller, Yang et al. 1993). Commercially available EIA diagnosis kits for serological testing of Hepatitis C virus successfully employ an array of recombinant core nonstructural HCV proteins (Majid and Gretch 2002). A Dengue virus diagnostic immunochromatographic assay successfully uses four envelope glycoproteins purified from stably transfected S2 cells, and mimics to a great extent whole virus preparations in terms of sensitivity and specificity for diagnosis of primary and secondary dengue infections (Cuzzubbo, Endy et al. 2001; Huang, Huang et al. 2001). Successful Japanese encephalitis virus diagnosis depends on premembrane/membrane and envelope proteins of JEV expressed by a vaccinia/HeLa system (Konishi, Mason et al. 1996).

The use of recombinant proteins in serodiagnosis is definitely not without challenges. Production and purification of large amounts of protein is usually a labor-intensive, multi-step process and may be costly. The authenticity of the recombinant agent may be affected by technical necessities such as protein denaturing and refolding, which is likely to compromise the structure of conformational epitopes. Antigen-antibody affinity may be reduced by slight changes in conformation, especially when the antigen-antibody interaction surface area is large. Purification is also hindered by unavailability of matching antibodies, and the need for addition of synthetic tags to aid purification and/or

identification. The enzymatic removal of these tags is technically arduous and presents higher chances of altered antigenicity and immunogenicity. The choice of the production system, whether prokaryotic or eukaryotic, also raises questions of post-translational processing and glycosylation modes in these systems, especially in proteins where this is known to affect their function or reactivity to antibodies.

In the case of B virus, a BSL-4 pathogen, the use of recombinant antigen based immunoassays for diagnosis carries numerous benefits. Glycoproteins embedded in the lipid envelope of herpesviruses have been used as antigens in serodiagnosis due to their availability to the immune system, and thus, the frequent presence of antibodies against them in sera of infected animals and/or humans. Bacterial expression of herpesvirus glycoproteins has been frequently employed for its high yields; however, immunogenicity has been poor due to improper folding, flawed disulfide bond formation, and lack of glycosylation (van Drunen Littel-van den Hurk, Parker et al. 1993). Baculovirus/insect cell expression systems have been widely used for their good yield, ease of purification and convenient cell culture techniques. Glycoproteins of herpesviruses expressed in these systems have in general shown a good degree of immunogenicity, retained antigenicity, and bioactivity (Sisk, Bradley et al. 1994; Nicola, Willis et al. 1996).

Glycoprotein D (gD), one of eleven glycoproteins identified in the B virus envelope, is among the main surface antigens shown to elicit antibodies in sera of infected animals. Glycoprotein D was immunoprecipitated by antisera to B virus, as well as to SA8, HSV-1 and HSV-2 (Eberle, Black et al. 1989). Immunization of Japanese macaques with expression plasmids encoding B virus gD induced humoral and cellular immunity (Hirano, Nakamura et al. 2002). Moreover, homologues of B virus gD such as glycoprotein D of HSV-1 and HSV-2 are immunodominant envelope proteins that can elicit humoral and cellular immunity in animals and human individuals and were considered good candidates at that time for vaccine design (Martin and Rouse 1987; Heber-Katz 1998;

Stanberry, Spruance et al. 2002; Bernstein, Aoki et al. 2005; Quenelle, Collins et al. 2006). HSV-1 gD shows 69 % nucleotide sequence identity and 56-58 % amino acid identity to B virus gD (Bennett, Harrington et al. 1992; Perelygina, Zhu et al. 2003). HSV-1 gD binding to the host receptor (HVEM) prompts NF- κ B activation resulting in virus-directed inhibition of infected cell apoptosis (Medici, Sciortino et al. 2003). Glycoprotein D binding to primary cells is involved in induction of IFN- γ , a protective host response during infection (Pollara, Jones et al. 2004). Another homolog of B virus gD, BHV-1 gD, was shown to confer protection from infection in immunized cattle and the immune response was found to be stronger than that induced by gB or gC (van Drunen Littel-van den Hurk, Parker et al. 1993).

Glycoprotein D is one of four envelope glycoproteins that mediate entry of HSV-1 into target cells. Three receptors have been identified: HVEM, nectin-1, and 3-O-sulfated heparan sulfate. Differential use of these receptors has been proposed to account for entry into various cell types (Richart, Simpson et al. 2003; Tiwari, Clement et al. 2005; Tiwari, Clement et al. 2006; Reske, Pollara et al. 2007). B virus gD is a type I membrane glycoprotein that consists of 370 amino acids in its mature form, with an N-terminal domain of 316 amino acids and a transmembrane domain between amino acids 317-339. Two possible N-linked glycosylation sites on residues 94 (NLTV) and 262 (NATR) were identified in glycoprotein D of prototypic cynomolgus and macaque B virus isolates (Bennett, Harrington et al. 1992; Perelygina, Zhu et al. 2003). Eight of nineteen clinical strains of B virus have an additional N-linked glycosylation site on amino acid 122, a site known to be N-glycosylated on HSV-1 gD (Perelygina, L, personal communication). The binding region of HSV-1 gD to its receptor HVEM has been defined by crystallographic analysis and found to be amino acids 1-32 (Carfi, Willis et al. 2001; Connolly, Landsburg et al. 2003). Potential binding residues to another receptor, nectin-1, are under study (Connolly, Landsburg et al. 2005). Residues past amino acid 260 are believed to block binding of gD to HVEM and nectin-1 by acting as profusion domains (260-285 and 285-

310) which keep gD in a “closed” conformation prior to encounter with its receptors (Fusco, Forghieri et al. 2005). A conformational change follows binding of gD to its receptor, bringing the virus in closer contact to the cell surface (Krummenacher, Supekar et al. 2005). When B virus gD is compared to gD homologs of closely related viruses, most of the conserved residues are found in the ectodomain (Bennett, Harrington et al. 1992; Pereyagina, Zhu et al. 2003) .

Upon mapping of linear epitopes of B virus gD, an immunodominant epitope in the C-terminus of B virus gD (362-370), located in the cytoplasm of infected cells, was identified (Pereyagina, Patrusheva et al. 2002). This epitope exhibited high reactivity to sera from B virus-infected humans and macaques, at the same time displaying minimum cross-reactivity to HSV-1 and HSV-2 positive sera. It is likely that the immune response directed toward this epitope is a result of antigen processing and presentation given the cryptic nature of the epitope in the context of intact, infected cells. Investigators observed, however, that 41% (31/75) of antibody positive macaque serum samples reacted more strongly with the ectodomain of mammalian recombinant gD (gD 324t) than to the gD 362-370 epitope. This reactivity decreased upon denaturing gD 324t, indicating the presence of immunogenic conformational epitopes on the ectodomain of B virus gD. Serum samples from infected humans and macaques showed consistently decreased reactivity with denatured recombinant gD in contrast to the non-denatured counterpart produced in CHO cells.

Antisera produced by DNA immunization of rabbits against gB, gC, gD, or gE were tested against B virus-infected cell lysates using ELISA, western blot, and immunoprecipitation assays. Remarkable reactivity was observed with gD antisera, second only to that of gB, especially in assays using non-denatured antigen. Although gB antisera in rabbits had a higher titer, gD antisera showed lower cross-reactivity to HSV-1 and HSV-2 gD. When gD rabbit antisera were tested against denatured CHO gD, reactivity was again minimal. Glycoprotein D showed lower cross-reactivity with HSV-1 and HSV-2 antisera,

suggesting it may be a good candidate for differential diagnosis of B virus in humans and animals with pre-existing antibodies to HSV-1, HSV-2, or other closely related alphaherpesviruses, especially when the assay is based upon detection of conformational epitopes.

In a separate report, B virus full length gD was expressed in Cos7 cells and the cell lysates were used as antigen in western blots and dot blots to test sera from infected monkeys (Tanabayashi, Mukai et al. 2001). Eight B virus positive macaque serum samples were tested by western blot; only three showed substantial immunoreactivity with gD-expressing cells. Another three sera reacted equally with cells transfected with gD-expressing plasmid and those transfected with vector DNA. The last two showed less immunoreactivity than that towards cells containing vector DNA. A secreted form representing the ectodomain of gD tested in its nondenatured form in a dot blot assay of 61 positive sera and 36 negative sera resulted in 100 % sensitivity and specificity. It was concluded that expressed gD lost its antigenicity during the western blot procedure, confirming the above results obtained in our lab. In addition, these results concurred with earlier studies with HSV-1 showing that envelope glycoproteins had denaturation sensitive epitopes (Eberle, Mou et al. 1985). Similar results were obtained in gD-based HSV-1 serodiagnosis: ELISA using HSV gD resulted in greater detection sensitivity than western blotting and had a better quantitative potential for measuring the antibody response (Bernstein, Garratty et al. 1985). The evidence therefore indicates clearly that when gD is to be used for serodiagnosis, it needs to be presented in a non-denatured form where conformational epitopes of the ectodomain are conserved. The focus of the work that follows was designed to investigate the utility of recombinant B virus gD for serodetection of B virus antibodies in samples of macaque and human sera.

In this work, we expressed a truncated form of B virus gD, consisting of the first 287 amino acids of the extracellular domain, using a baculovirus vector designed for expression in Sf9 insect cells. We purified and characterized gD287 and tested this novel

reagent as a diagnostic antigen by indirect ELISA. Infected and non-infected macaque sera used in testing were obtained from the repository of the National Herpes B Virus Resource Center in Atlanta, Georgia.

II. MATERIALS AND METHODS

A. Cells and Viruses

Sf9 cells (ATCC CRL-1711, ovary, fall armyworm, *Spodoptera frugiperda*) were propagated in Sf-900 II SFM medium (Invitrogen, Carlsbad, CA) supplemented with Penicillin-Streptomycin at 50 I.U./ml. Cells were grown in monolayer or suspension cultures at 27°C in a non-humidified air environment and maintained in log phase at a density of 2×10^6 cells/ml in suspension or 10^5 cells/cm² in monolayers in T-75 and T-175 flasks (Nalge Nunc International, Rochester, N.Y.). Viability was maintained at greater than 95% and regularly checked by trypan blue exclusion. Two recombinant baculoviruses (nuclear polyhedrosis virus) expressing truncated forms of gD were used: Bac-gD324 and Bac-gD287, expressing the first 324 and 287 N-terminal amino acids, respectively. Both viruses were constructed using the BAC-TO-BAC baculovirus expression system (Invitrogen, Carlsbad, CA). The plasmid expressing Bac-gD324 was previously constructed by colleagues in our lab while Bac-gD 287 construction was part of this work and is explained below in detail.

B. Construction of Bac-gD287 recombinant virus

1. PCR and cloning into pCR-XL-TOPO

The gD ORF (nucleotides 73-933) of B virus strain E2490 was amplified by PCR with the primers gD73F-BAM 5'-**GGATCCGAGTACGTGCCGGTGGAGC**-3' and gD933F-XBA 5'-**TCTAGAGCCCTGGATGGTGACGTCG**-3' (Sigma, St Louis, MO)

using 50 ng of B virus genomic DNA as template. The PCR reaction was carried out in 1X PCR buffer with 0.2% DMSO, 200 μ M of each dNTP, 200 μ M of each primer, and 2 units of HotStar Taq DNA polymerase (Qiagen, Valencia, CA) in Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA). The following amplification conditions were used: 15 minutes at 95°C for activation of Taq DNA Polymerase, followed by cycling 35 times for 20 second at 95°C / 20 second at 65°C. The PCR product was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and cloned into the plasmid vector pCR-XL-TOPO (Invitrogen, Carlsbad, CA). Since the multiple cloning site in pCR-XL-TOPO was inside the lacZ α ORF, cloning of the PCR-amplified fragment into the vector disrupted the expression of β -galactosidase, resulting in the appearance of white recombinant colonies of transformed bacteria on X-gal plates. The TOPO vector also contains kanamycin and zeocin resistance ORF's, allowing selection of transformed cells on antibiotic-containing media.

The cloning procedure was as follows; 4 μ l of purified PCR product was added to 1 μ l of pCR-XL-TOPO vector and incubated for 5 minutes at room temperature. An aliquot of 6X TOPO Cloning Stop Solution (1 μ l) was added subsequently and mixed for several seconds, then 2 μ l of the cloning reaction was transformed into One Shot Top 10 chemically competent *E. coli* according to manufacturer's instructions. Transformed bacteria were subsequently plated on LB plates containing kanamycin (50 μ g/ml), 100 μ M isopropylthiogalactoside (IPTG), and X-Gal (16 μ g/ml), then incubated overnight at 37°C. White colonies were picked and grown in 2 ml of LB medium supplemented with kanamycin. The gD 287-TOPO recombinant plasmid DNA was extracted from the cell pellets using the alkaline lysis method, precipitated with ethanol, and dissolved in 100 μ l

of TE buffer (Qiagen, Valencia, CA). Plasmid size was verified by electrophoresis on a 1% agarose gel and recombinant clones with the proper insert size were confirmed by enzymatic digestion with EcoRI followed by gel electrophoresis. Recombinant plasmids were purified using QIAquickspin purification kit and inserts sequenced using BigDye terminator sequencing kit (ABI PRISM).

2. Transposition of the gD 287 segment into baculovirus shuttle vector

Recombinant baculovirus expressing gD 287 was constructed using the BAC-TO-BAC baculovirus expression system (Invitrogen, Carlsbad, CA). The system used site-specific transposition of the gene of interest from a donor plasmid to a baculovirus shuttle vector (bacmid) in DH10Bac competent *E. coli* (Figure 1). Transposition functions were provided in trans by a helper plasmid encoding transposase. This maximized recombination efficiency over traditional recombination methods and reduced the need for virus purification assays. Transposition disrupted a segment expressing lacZ on the bacmid, allowing for selection of white colonies on lactose-providing media.

The segment expressing gD 287 was transferred from the gD-TOPO plasmid into a donor plasmid pFBMV5H, a modified form of pFastBac donor plasmid (Invitrogen, Carlsbad, CA). The plasmid pFBMV5H contains an additional honeybee mellitin secretion signal, a V5 epitope (**Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr**) expression segment for easy detection of the recombinant protein with anti-V5 monoclonal antibodies, and a hexahistidine tag expression segment to facilitate purification on metal-affinity resins. The plasmid pFBMV5H also contains ampicillin and gentamicin resistance genes. Both gD 287-TOPO and pFBMV5H were digested with XbaI and BamHI and gel-purified using the QIAquick gel extraction kit. The gel-purified

gD 287 fragment and pFBMV5H were coprecipitated with ethanol. The pellet was dissolved in 7 µl of molecular grade water by gentle pipetting. A Fast-Link DNA ligation

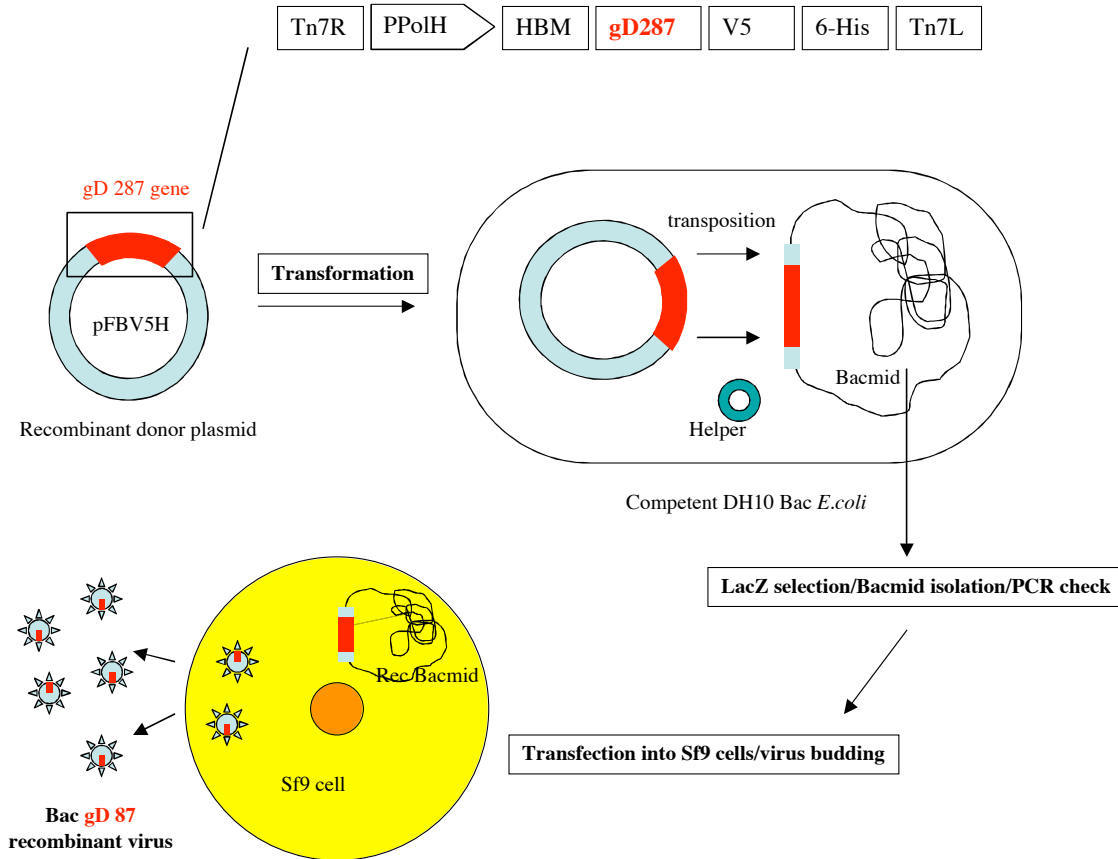


Figure 1. Schematic representation of BAC-TO-BAC baculovirus expression system (Invitrogen, Carlsbad, CA) for production of gD recombinant baculovirus. Schematic representation is not to scale.

kit was used for ligation of gD-287 into pFBMV5H in the presence of 10 mM ATP. An aliquot (50 µl) of chemically competent Nova Blue *E. coli* was then transformed with 2 µl of the gD 287-pFBMV5H ligation mixture. Transformed cells were incubated in S.O.C

medium for one hour at 37°C with gentle shaking, and subsequently grown overnight on agar plates with 50 µg/ml ampicillin. Colonies were picked and grown in suspension overnight at 37°C in LB/amp. Subsequently, the gD/pFBMV5H plasmid was purified from the cell pellets by ethanol precipitation, and resuspended in 100 µl of TE buffer.

Transposition into the baculovirus shuttle vector was accomplished by transforming 50 µl of *E.coli* DH10Bac competent cells with 4 µl of gD 287-pFBMV5H. The cells were grown in 450 µl of S.O.C medium for 4 hours, then overnight on S-gal agar plates with 50 µg/ml kanamycin, 7 µg/ml gentamicin, and 10 µg/ml tetracycline. Since colonies produced by DH10Bac transformed cells are generally small, white colonies were selected and streaked on a similar plate overnight for colony color confirmation. Cells from confirmed white streaks were grown in suspension and DNA was isolated by the alkaline lysis method. Briefly, cell pellets were treated with 300 µl of each of the buffers P1, P2, and P3 (Qiagen, Valencia, CA). After clearing solid protein aggregates and bacterial DNA, recombinant bacmids were precipitated with ethanol, dried, and dissolved in 60 µl of TE buffer. The size of the resulting recombinant bacmids was checked by electrophoresis on a 0.8% agarose gel (bands migrating more slowly than 12 kb; 90V for 45 minutes).

To confirm proper transposition, we subjected the bacmid to PCR, using one gene-specific primer (gD 73F-BAM) and an antisense bacmid-specific primer (M13/pUC Reverse primer as described above with a longer final extension step (40 seconds at 72° C). The PCR product was run on a 0.8% agarose gel for size confirmation.

3. Transfection and recombinant virus amplification

For transfection of recombinant bacmids into Sf9 cells, Cellfectin Reagent

(Invitrogen, Carlsbad, CA), which is a liposome formulation containing cationic and neutral lipids in membrane-filtered water, was utilized according to the manufacturer's recommendations. This reagent interacts spontaneously with DNA to form lipid-DNA complexes that are subsequently taken up by cells. The insect Sf9 cells were grown in a six well microplate at a density of 1×10^6 cells/well. Recombinant bacmid DNA (5 μ l) was suspended in 100 μ l of Sf-900 II SFM (serum free medium; Invitrogen, Carlsbad, CA). In parallel, 6 μ l of Cellfectin Reagent was diluted with 100 μ l of Sf-900 II SFM. The two preparations were then mixed gently and left for 45 minutes at room temperature to form the DNA-lipid complexes. Cells were washed once with antibiotic-free Sf-900 II SFM to remove detached cells, the transfection mixture was diluted with 0.8 ml of Sf-900 II SFM, and 100 μ l of the diluted sample was added to each well. Cells were incubated with the transfection mixture for 5 hours at 27° C, after which the transfection mixture was removed and cells were overlaid with 2 ml of Sf-900 II SFM supplemented with antibiotics. Cells were then grown for 72 hours to allow for viral budding. The medium was collected from each well and titrated on Sf9 cell monolayers for plaque production.

4. Viral plaque assay

Viral plaque purification methods were used to ensure that the recombinant virus stock originated from a single baculovirus particle. The Sf9 cells in six-well plates were incubated with 0.5 ml of ten-fold serially diluted transfection mixture (10^{-1} to 10^{-4}). Infection was allowed to proceed for 1 hour at room temperature with gentle rocking. An agarose overlay was prepared using 1.3 X Sf 900 concentrated serum free medium 1:3 in melted 4% agarose (Invitrogen, Carlsbad, CA). Virus was then removed and cells were overlaid with agarose mixture, allowed to solidify, and incubated for eight days in a

humidified incubator at 27° C. Plaques of baculovirus-infected cells were then picked with sterile Pasteur pipettes and resuspended each in 1 ml of 3% FBS in Sf-900 II SFM.

5. Recombinant baculovirus amplification

Log-phase Sf9 cells were incubated in a six-well plate at 1×10^6 cells/well with 0.5 ml/well of single viral plaque suspensions. One hour after the addition of virus, cells were supplemented by 2 ml per well of 3% FBS in SFM. Protein production and cytopathic effects of baculovirus infection were monitored every 24 hours. At 96 hpi, the supernatant was collected from each well, cleared from cell debris by centrifugation, and stored under sterile conditions. Dot blot and western blot analysis using monoclonal anti-V5 antibody was employed to select the plaques with the highest production of gD 287. We used monoclonal anti-V5 antibodies to detect the presence of gD 287 in the collected stocks. After comparing production by different plaques, the optimum clone was selected for amplification. In 75 cm² flat-bottom flasks, 1.2×10^7 cells in subconfluent monolayers were infected with 0.5 ml of the virus stock diluted with 3 ml of 3% FBS SFM. After one-hour incubation at 27° C with gentle rocking to enhance virus attachment, cells were supplemented with 8 ml of media and grown for 72 hours. The supernatant containing recombinant virus was clarified by centrifugation and subsequently virus was titrated using Sf9 cells (10^{-4} to 10^{-8} dilutions) as previously described. Another round of amplification was performed by infecting 100 ml of suspension cell culture at MOI of 0.1 PFU/cell, and the supernatant collected between 72 and 96 hpi or when viability dropped below 60%. The supernatant containing recombinant virus was collected and clarified by centrifugation at 800 g for 20 minutes at 4°C and stored refrigerated in the dark. Viral titer was determined by the viral plaque assay.

C. Expression and purification of gD by nickel-chelate affinity chromatography

For recombinant gD production, Sf9 suspension cultures (125 and 250 ml) in log phase growth were infected with recombinant baculovirus stocks at MOI = 5 and the supernatant was collected by centrifugation (1000 g, 4°C, 20 min) either at 72 hpi, or when cell viability dropped below 70%. The supernatant was then filtered (0.45 µM) and subsequently concentrated, desalted, and dialyzed against chromatography buffer (40 mM sodium phosphate, 0.3 M NaCl, 10% glycerol, pH 7.8) using a Pellicon XL tangential flow filter (10 kDa exclusion) on the TFF Labscale system (Millipore, Bedford, MA).

Recombinant gD was purified by nickel chelate affinity chromatography on the *Akta* FPLC system (Amersham Biosciences, Piscataway, NJ). Although the purification at hand was not overly time consuming to warrant the use of FPLC, the long-term application of this project was production of milligrams of recombinant proteins for use in serodiagnosis. The standardization of a method using automated fast purification performance instead of the traditional gravity flow method was therefore undertaken as a part of this work. Generally, purification under high pressure results in a highly pure product while the automated detection of the purified reagent's quality, as provided by the FPLC workbench, makes purification easier to automate and standardize for batch production. A highly cross-linked agarose Hi Trap Chelating column (1ml) (Amersham Biosciences, Piscataway, NJ) was loaded with Ni⁺² using 1 ml of 0.1 M NiSO₄, washed with 5 column volumes (CV) of distilled water, and mounted on *Akta* FPLC system. Two buffers were prepared: buffer A (40 mM sodium phosphate, 0.3M NaCl, 10% glycerol,

pH 7.8) and buffer B (buffer A + 0.5 M imidazole). The operating software (Unicorn 3.2, Amersham Biosciences) was used to create a method involving the following steps:

- Equilibration of the column with 5 column volumes (CV) of buffer A.
- Sample loading into the column at 1 ml/min using superloop pump.
- Washing unbound gD and nonspecifically bound proteins with 5 CV of 15mM imidazole (3% buffer B in buffer A).
- Elution of gD using a step gradient of 250 mM imidazole. Eluted fractions were collected in 0.5 ml portions at UV absorbance peak with a start slope of 100 mAU/min and end slope of 75 mAU/min at A_{280} .
- Cleaning of the column with 8 CV of buffer B (500 mM imidazole).
- Re-equilibration with 8 CV of buffer A.

Eluted peak fractions were stored in polypropylene vials at -20°C for further analysis.

D. Recombinant gD characterization

1. SDS-PAGE

Nickel chelate affinity chromatography eluted fractions were tested by 10% SDS-PAGE. Samples were boiled with 2X SDS sample buffer (20 mM Tris-HCl, pH 6.8; 0.2% 2-ME, 4% SDS, 20% glycerol, 0.001% bromophenol blue) at 95°C for 5 minutes and run on 10% SDS-PAGE gels in Tris- Glycine-SDS buffer for 90 minutes at 110 V. Gels were dried and scanned. The identity of recombinant gD was confirmed by immunoblot and western blot using rabbit gD antisera produced by DNA immunization at a dilution of 1:200 (Perelygina, Patrusheva et al. 2002), and pooled sera from B virus infected monkeys at a dilution of 1:100 (provided by the National B Virus Resource Center, Atlanta, GA).

2. Dot blot assay

An aliquot (1-2 μ l) from samples which represented different stages of purification were spotted on a nitrocellulose membrane, air dried, and blocked from one hour to overnight in Blotto (PBS, 0.1% Tween, 5% skimmed dry milk, 1% heat inactivated normal goat serum). The membrane was then incubated with antibodies against gD (e.g. anti-V5 MAb; 1:3500) diluted in Blotto for one hour. The membrane was washed thrice (5-10 minutes on an orbital shaker) and probed with a goat anti-human horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL). After washing as described above, the signal was developed using the enhanced chemiluminescence method (ECL) (Amersham Biosciences, Piscataway, NJ). Briefly, the membrane was incubated with ECL substrate mixture for one minute, and then exposed to Kodak X-omat LS film (Sigma, St. Louis MO).

3. Western blot

Samples were fractionated on SDS-PAGE as described, and then transferred to a 0.45 μ m nitrocellulose membrane (Invitrogen, Carlsbad, CA). The membrane was then blocked from two hours to overnight, probed with antibody (see results for use of specific antibody), and developed as described above (section 2).

4. Bradford assay for determination of protein concentration

Affinity-purified gD 287 protein concentration was determined using the Coomassie Plus protein assay kit (Pierce, Rockford, IL). The protocol is a modification of the Bradford method for determination of protein concentration (Bradford 1976). Bovine

serum albumin (BSA) standards were prepared at concentrations between 25 µg/ml and 2000 µg/ml using chromatography elution buffer as the diluent and the blank control. Coomassie Plus reagent was added and absorbance was read using an Ultraspec 3000 (Pharmacia Biotech, Sweden) spectrophotometer (A_{595}); The resultant data were plotted using a quadratic fit according to manufacturer's instructions and results tabulated using SoftMax Pro 3.0 (Molecular Devices, Sunnyvale, CA).

E. Testing macaque sera using recombinant gD ELISA

Maxisorp 96-well plates (Nalge Nunc International, Rochester, N.Y.) were coated with purified gD (12.5 – 200 ng/well) diluted in 20 mM Tris-HCl pH 8.0, incubated with gentle shaking for one hour at room temperature, and kept overnight at 4°C. Next morning, the plates were washed with 20 mM Tris-HCl pH 8.0 and blocked with 200 µl/well of ELISA-Blotto (BBS, 2.5% skimmed dried milk, 2.5% liquid gelatin) for 1h at room temperature. The blocking buffer was removed; sera (50 µl/well) to be tested (diluted in ELISA-Blotto) were added (1h, room temperature, shaking). After washing, an aliquot of alkaline phosphatase-conjugated goat anti-human IgG Fc fragment (Sigma, St. Louis MO) was added at 50 µl/well (1:4000) (1h, room temperature, shaking). Substrate p-Nitrophenyl phosphate (pNPP) (1mg/ml, 200 µl/well) (Sigma, St. Louis MO) in buffer (1M diethanolamine, 0.5 mM MgCl₂, pH 9.8) was used. After incubating the microplate at room temperature for 25 minutes, the reaction was stopped by the addition of 50 µl/well of 3N NaOH. OD readings were determined at A_{405} on a Spectra Max 250 automatic plate reader (Molecular Devices, Sunnyvale, CA). Pooled sera from B virus infected and uninfected rhesus monkeys were included in each plate as positive and negative controls. All samples were run in triplicates and the mean OD values

calculated. Statistical analysis of diagnostic potential was performed using GraphPad Prism software version 4.00 for Macintosh (GraphPad Software, San Diego California USA, www.graphpad.com) to assess the predictive potential of the assay based on results from B virus total antigen testing.

III. RESULTS

Construction of recombinant baculovirus expressing gD 287

The DNA segment encoding the first 287 amino acids of the gD ectodomain of B virus was amplified by PCR, cloned into the pCR-XL-TOPO vector, and transformed into *E.coli*. The size of the recombinant gD-TOPO plasmid including the insert was ~4.2 kb as determined by electrophoresis on a 1% agarose gel. Digestion of the recombinant gD-TOPO plasmid with EcoRI resulted in two fragments migrating at 3.5 kb and 0.9 kb on an 1% agarose gel. Subsequently, DNA sequencing confirmed that the cloned gene matched nucleotides 73-933 of the gD ORF of B virus. The cloned gene segment was enzymatically excised from the TOPO vector using XbaI and BamHI and ligated into pFBV5H after gel purification. The resulting gD 287-pFBV5H recombinant plasmid was propagated in *E.coli*, purified and sequenced using the PFB-1 primer 5'-TATTCCGGATTATTCATACC-3' and the PFB4251R primer 5'-CCTCTACAAATGTGGTATG-3'. gD 287-pFBV5H was used as a donor plasmid for transposition of gD 287 into the baculovirus genome contained in *E.coli* DH10Bac competent cells (Invitrogen, Carlsbad, CA). Positive colonies were selected for propagation and purification of recombinant bacmid DNA. Successful transposition of gD 287 into baculovirus genome under the polyhedrin promoter was confirmed by PCR using one gene-specific primer (gD 73F-BAM) and an antisense bacmid-specific primer

(M13/pUC Reverse primer). The amplified PCR product migrated as ~1.5 kb band on a 0.8% agarose gel. This band size corresponded to the cumulative size of gD 287 (861 bp), additional segments transposed from the pFBMV5H (458 bp) and the part of the bacmid (145 bp) from the M13R origination site (Figure 2). The recombinant bacmid was transfected into Sf9 cells using the Cellfectin transfection method according to the manufacturer's recommendation (Invitrogen, Carlsbad, CA). The resulting viral suspension had a titer of 10^4 PFU/ml as determined by viral plaque assay.

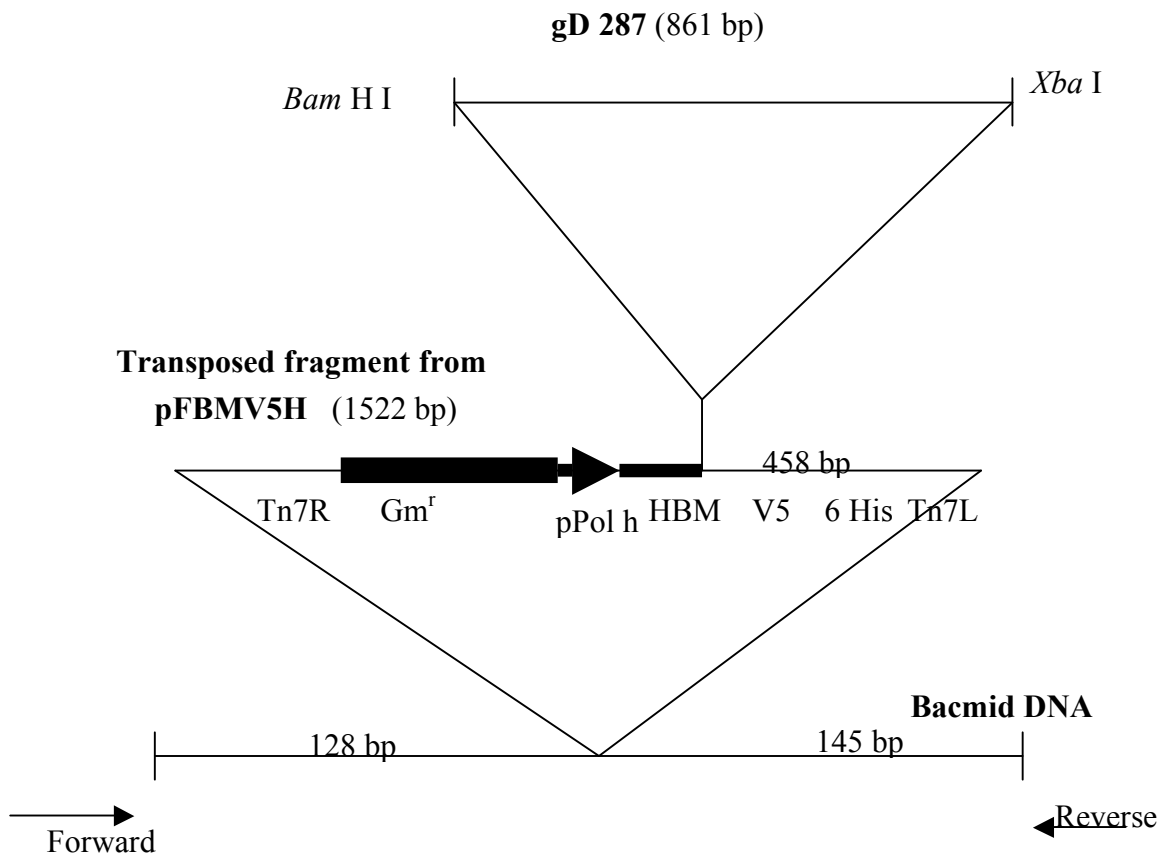


Figure 2. Schematic representation of gD 287 transposition into baculovirus genome. Transposition was confirmed by PCR-amplified fragment of ~1.5 kb (M13R and BAM73F). This is equivalent to the sum of transcribed fragments (145 + 458 + 861 bp).

Amplification of recombinant baculovirus expressing gD 287

Single recombinant virus plaques were picked, resuspended in Sf-900 II SFM media and amplified to obtain working virus suspension stocks. Single plaque suspensions were used to infect Sf9 cell monolayers in 6-well plates. Different wells were assayed for gD 287 production to select recombinants with the highest production potential. Cells or supernatants were boiled in SDS sample buffer and tested by immunoblotting for estimation of gD secretion efficiency. Virus amplification by infection of Sf9 monolayers and suspension cultures resulted in titers of 10^7 and 10^8 PFU/ml, respectively.

Optimization of recombinant gD production

To determine optimal multiplicity of infection, Sf 9 cell monolayers in a 6-well plate were infected with recombinant virus at MOI of 1, 3, 5, 6, or 10. Supernatant samples were collected at 24-h intervals post infection. To determine optimal sample collection time (high gD amount in supernatant, low degradation due to cell death), suspension cell cultures were infected with recombinant baculovirus using an optimum MOI of 5 (result of previous experiment) and supernatant samples collected at regular intervals (0, 24, 48, 72, 96, and 110 hours post infection). Samples were analyzed by western blot and dot blot using anti-V5 Mab (1:3500) (Figures 3 and 4). The major immunoreactive band of ~46 kDa observed in western blots of infected cells and supernatant was most likely full length gD 287 as determined by molecular weight comparison to full length gD taking into account possible glycosylation. A minor protein band of ~30 kDa that was detectable by 48 hpi, peaking by 72 hpi, was expected to be an intermediate processing form of gD 287 as were the various bands that appeared in the

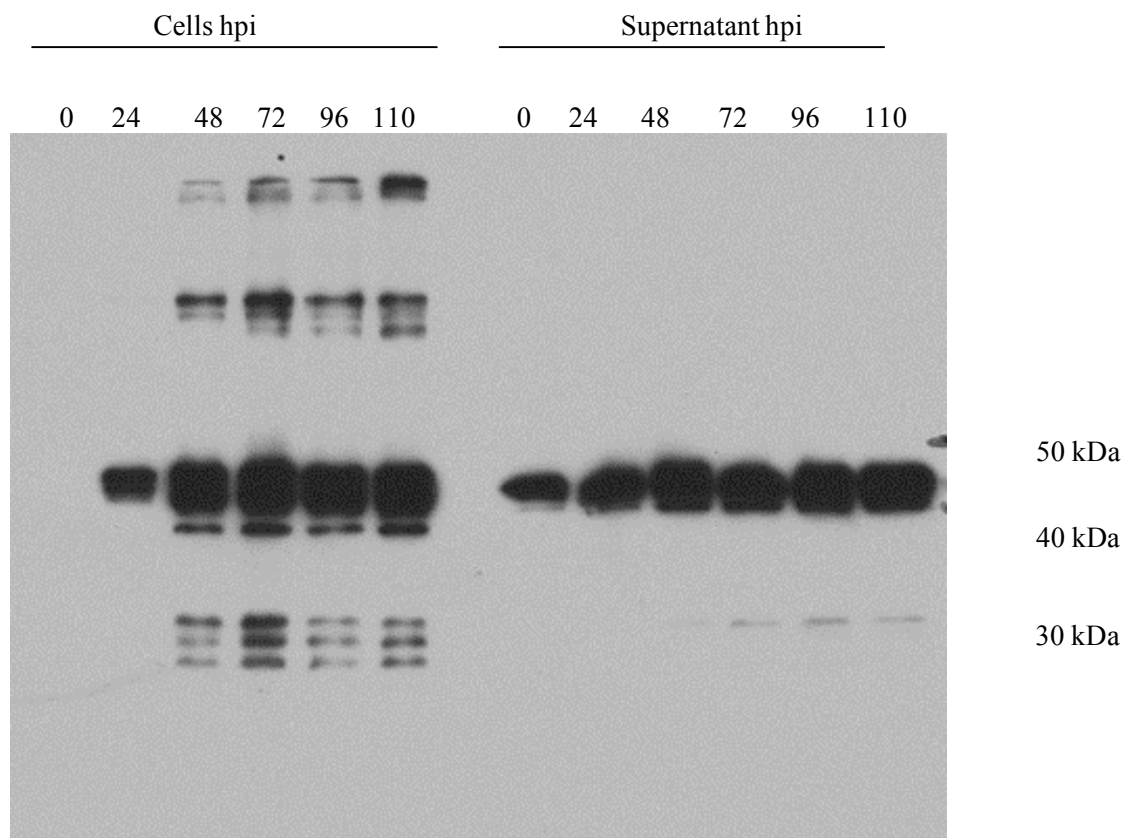


Figure 3. Western blot of gD 287 production over time. Cells and supernatants from Sf9 suspension cultures were infected with baculo-gD 287 at MOI=5 PFU/ml. Cells were diluted 10x compared to supernatants. 5µl of sample was run per lane and anti-V5 Mab was used 1:3500; middle lane contains sample loading buffer.

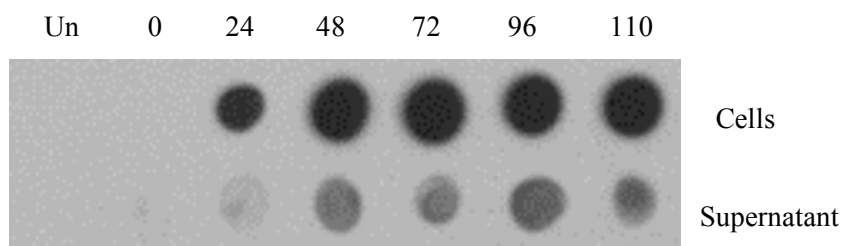


Figure 4. Immunoblot of gD 287 production over time. Cells and supernatant samples of baculo-gD 287 infection of Sf9 suspension cultures. 1 µl samples were probed by anti-V5 antibody (1:3500) and developed by ECL.

Affinity Purification of gD 287

cell lysate since the possibility of cross-reaction of the antibody with cellular proteins was minimal. According to the dot blot results, the recombinant gD 287 protein was secreted into the media by 24 hpi, with peak quantities being obtained between 48 and 72

hpi. Recombinant gD 287 protein, a fusion protein, was purified from the supernatant of infected cells using nickel-chelate affinity chromatography. The translated gD 287 was eluted with a 250 mM imidazole step gradient. Peak fractions were identified by UV absorbance readings on an *Akta* FPLC and confirmed by dot blot and western blot. The identity of gD 287 was confirmed using different antibodies: anti-V5 Mab, rabbit anti-gD serum, and B virus antibody positive and negative rhesus pooled sera (Figure 5). The recombinant gD 287 migrated as a single band (~46 kDa) on 10% SDS polyacrylamide gels. The purity of gD 287 was greater than 90% as shown by SDS-PAGE (Fig. 6). We obtained average yields of 5 mg of recombinant gD 287 per 1 liter of culture supernatant.

Efficacy of gD 287 rec-ELISA assessment for B virus serodiagnosis

The potential use of recombinant gD 287 in ELISA for diagnosis of B virus specific antibody in rhesus macaque serum was tested. A checkerboard titration was performed to determine the optimal amount of gD 287 for coating Maxisorp microtiter plates and the optimal dilution of macaque sera. Serial two-fold dilutions of gD 287 (200, 100, 50, 25, 12.5 ng/well) were used for coating.

B virus antibody negative or positive pooled sera (Figure 7 **a & b**) were used in two-fold dilutions ranging from 1:200 to 1:12.5. Based on the checkerboard titration results, 100 ng/well of gD 287 was used as a coating antigen and all sera were tested at 1:50 dilution; these parameters resulted in the maximum P/N ratio of 14.2 (Figure 8). To evaluate the efficiency of gD 287 as an antigen in serodiagnosis of B virus antibodies, we randomly picked rhesus macaque serum samples that were previously tested using total viral protein lysates of B virus in indirect ELISA and western blot assays.

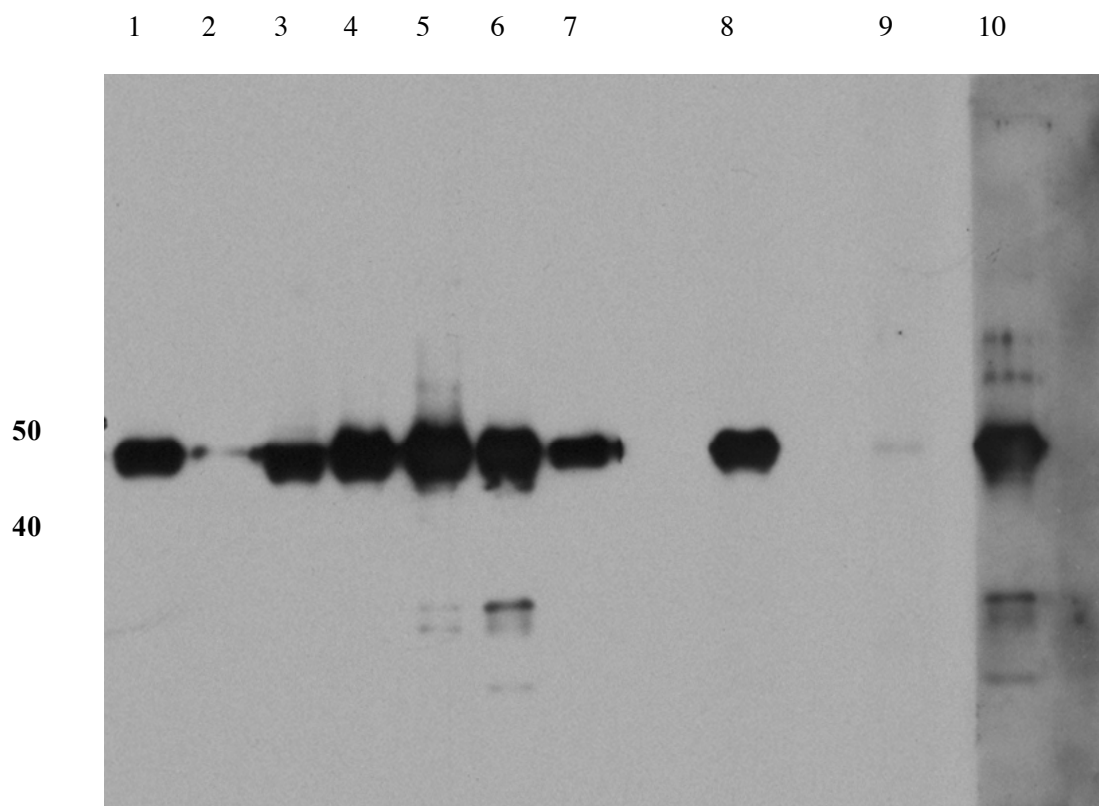


Figure 5. Western blot analysis of column fractions of the gD 287 purification. Lanes (1) through (7) were probed with anti-V5 Mab (1:3500). Lane (8) was probed with anti- CHO gD 324 (1:200). Lanes (9) and (10) were probed with negative and positive pooled rhesus sera (1:100), respectively. (1) Concentrated supernatant. (2) Flow through fraction. (3) 20 mM imidazole wash. (4) through (10) 250 mM elution fractions 3, 4, 5, 6, 6, 6, 6 (same fraction, 6, run with different antibodies as explained above).

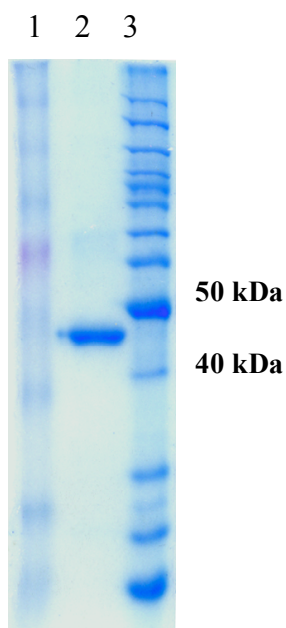


Figure 6. Assessment of gD purification by SDS-PAGE. Pre-stained molecular weight marker (lane 1), Nickel-affinity-purified gD 287 (3 mg/lane, lane 2), and 10 kDa protein ladder (lane 3) were separated on SDS-10% PAGE and stained with Coomassie blue.

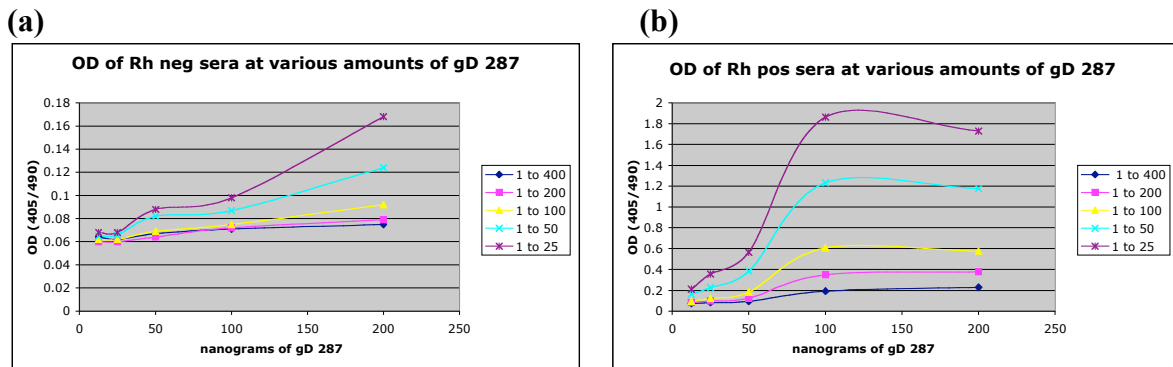


Figure 7. Titration curves of B virus antibody negative (a) or positive (b) rhesus pooled sera. OD values were plotted versus gD amount per well for each serum dilution.

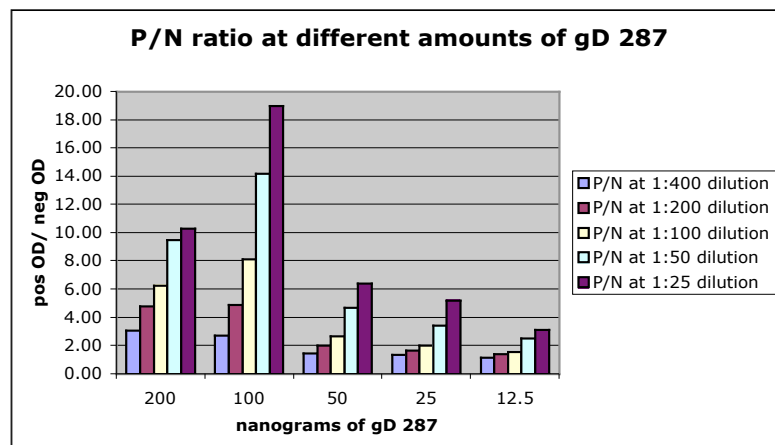


Figure 8. P/N ratios of positive and negative sera for various amounts of gD 287 adsorbed to ELISA plates.

Of the 94 sera, 72 samples classified as positive by standard whole virus antigen ELISA and western blot and 22 samples classified as negative were tested. There were 18 antibody positive samples from each of the four antibody-titer categories ($\leq 1:50$; $\leq 1:500$; $\leq 1:5000$; $>1:5000$), which were tested in triplicates alongside positive and negative pooled sera controls; OD readings are shown in Figure 9. A P/N value for each tested sample was generated as the ratio of the OD reading of this sample to the OD reading of pooled negative sera control in each plate. The P/N ratios from each titer range were then compared against each other and against those of the 22 antibody negative samples. The results were plotted cumulatively or by titer range group on a vertical scatter plot graph (Figures 10 and 11).

Diagnostic potential of gD 287 as an antigen was evaluated using Receiver-Operator curves (ROC) generated using GraphPad Prism software. The P/N cutoff value of 2.7 was obtained by calculating the mean P/N + 3SD from the 22 negative samples readings. When ROC analysis (area under curve =1 normally represents a perfect test) was applied to the cumulative positive/negative classification, we obtained low overall diagnostic potential manifested by area under curve = 0.5900 ($p=0.2032$) (Figure 12). To test the diagnostic potential of recombinant gD 287 in different titer groups, ROC analysis was applied to each titer group separately. The area-under-curve values and p values (Table 1) revealed the test was potentially efficacious for sera with antibody titers between 1:500 and 1:5000, and highly efficacious for sera with titers higher than 1:5000. As shown in Table 1, the sensitivity of the recombinant-based assay was 88.9 % (p value < 0.0001) for sera previously established to have titers greater than 5000. For sera with

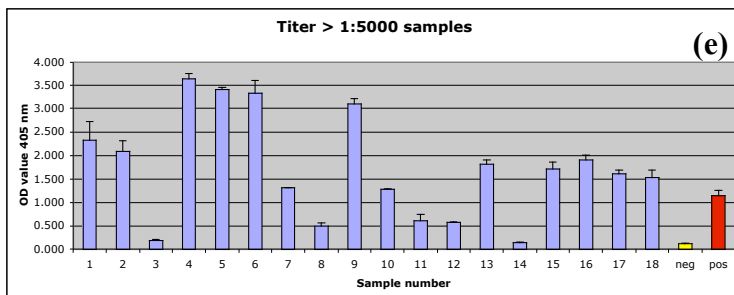
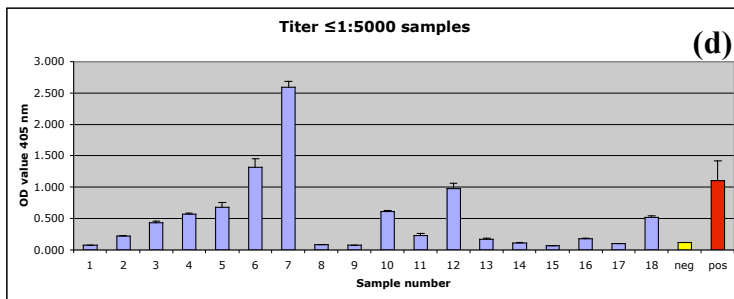
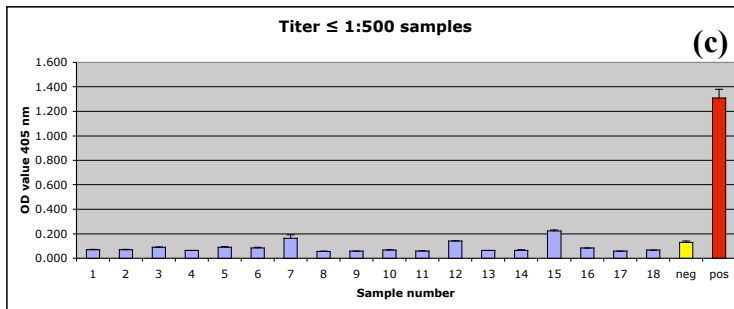
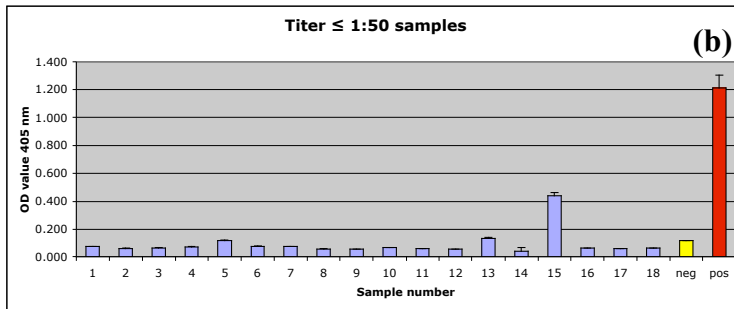
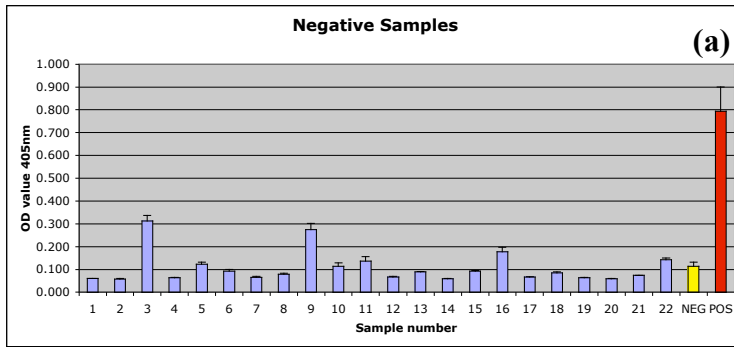


Figure 9. ELISA reactivity of individual serum samples from rhesus macaques with gD 287 recombinant antigen. Plates were coated with 100 ng of gD per well and reacted with either antibody negative samples (panel a) or with antibody positive samples from the selected titer groups (panels b-e)

Normalized Rec gD 287 ELISA by POS/NEG

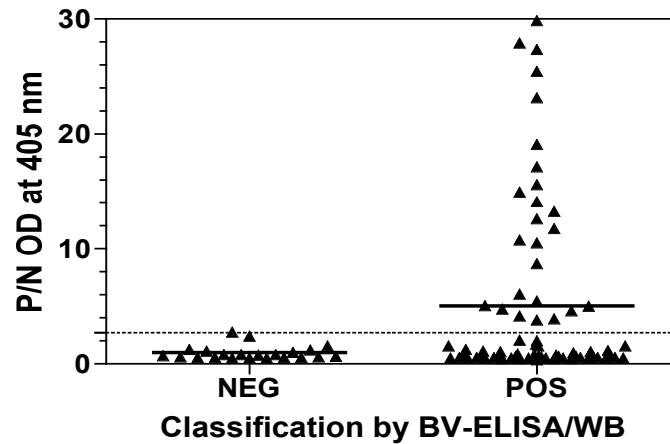


Figure 10. Distribution of P/N values for 22 negative and 72 positive samples. The dotted cutoff line is equivalent to the mean of negative serum P/N readings plus 3 SD. The means for negative and positive P/N values are 0.94 ± 0.60 and 5.02 ± 7.62 respectively.

Normalized Rec gD 287 ELISA by Titer Group

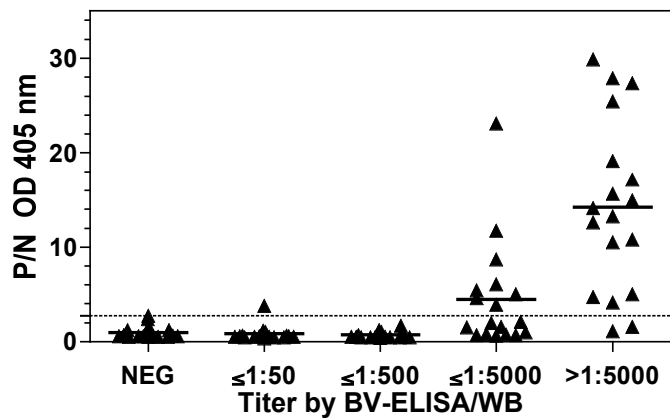


Figure 11. Distribution of P/N ratios of samples in various titer groups. Dotted cutoff line represents mean of NEG serum P/N readings + 3 SD. The means for each column from left to right are 0.94 ± 0.60 , 0.79 ± 0.77 , 0.67 ± 0.34 , 4.46 ± 5.61 , and 14.17 ± 9.08 .

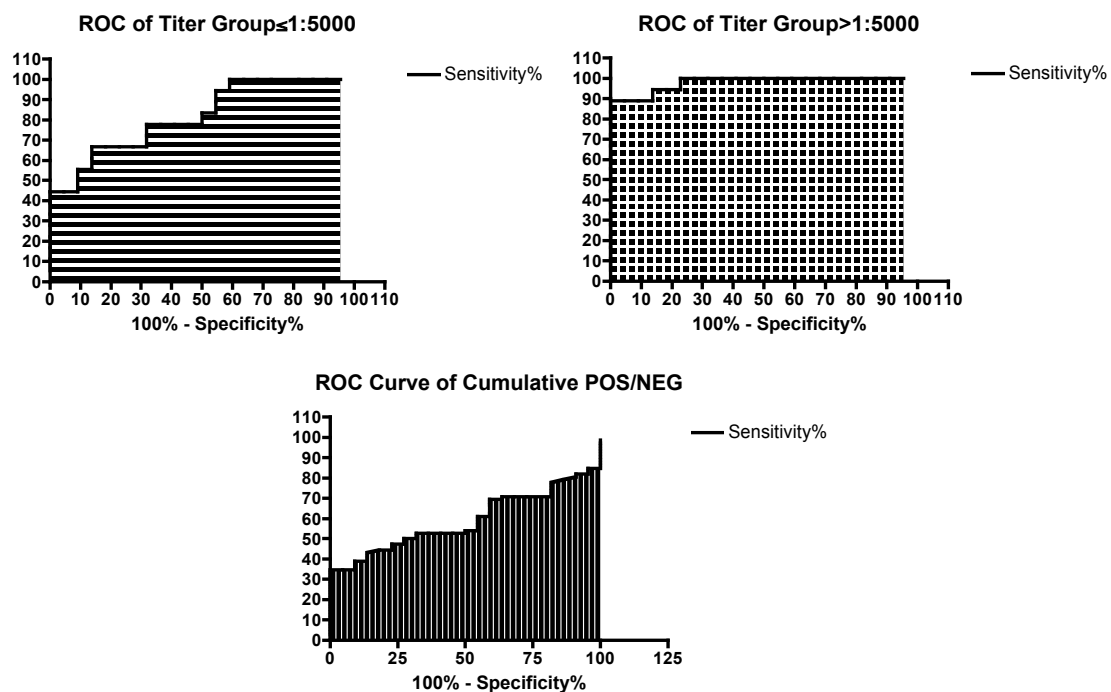


Figure 12. ROC curves representing sensitivity and specificity of each titer group and cumulative samples. The two lowest titer ranges are not represented since the test bears no diagnostic value for these groups with titer lower than 500.

	<i>Titer</i> $\leq 1:5000$	<i>Titer</i> $> 1:5000$	<i>Cumulative</i> <i>POS</i>
<i>Area under</i> <i>ROC</i>	<u>0.818</u>	<u>0.980</u>	<u>0.590</u>
<i>Std. Error</i>	0.066	0.017	0.057
<i>Cut Off value</i>	2.7	2.7	2.7
<i>95% CI</i>	0.688 to 0.948	0.946 to 1.014	0.478 to 0.702
<i>P value</i>	0.0006	< 0.0001	0.2032
<i>Sensitivity</i>	44.4	88.9	34.7
<i>95 % CI</i>	21.5% to 69.2%	65.3% to 98.6%	23.9% to 46.9%
<i>Specificity</i>	95.4	95.4	95.45
<i>95% CI</i>	77.2% to 99.9%	77.2% to 99.9%	77.2% to 99.9%

Table 1. ROC analysis of positive serum samples: analysis of specificity, sensitivity, and area under ROC of 72 samples tested by gD 287 rec ELISA. The two lowest titer ranges are not represented since the test bears no diagnostic value for these groups with titer lower than 500.

titers greater than 500 but lower than 5000, the sensitivity was 44.4% (p value < 0.0006).

The ROC analysis could not be used to calculate sensitivity values for sera with titers less than 500 because the reading values of tested samples were very close to those of negative controls. The OD ratio of pooled antibody positive serum to that of antibody negative serum was between 7.0 and 10.4 (average = 9.3). Two sera from the highest titer group tested negative at the set cutoff. No positive signal was detected for any of the sera in the two lowest titer groups.

Discussion:

This work culminated in the production and testing of a novel reagent to assess B virus-specific IgG antibody detection efficacy in macaque sera. An indirect ELISA was employed using as antigen a truncated form of B virus recombinant gD ectodomain, gD 287, generated using recombinant baculovirus. Macaque sera obtained from the repository of the National Herpes B Virus Resource Center (Atlanta, GA) had been previously tested using whole B virus antigen in western blot and ELISA. The good sensitivity of the assay for previously established high titer sera (>5000) showed that the recombinant gD 287 reacted readily with antibodies produced in the course of natural infection. The absence of false positives by this assay indicates minimum cross-reactivity of gD 287 with other antibodies in the macaque sera, but it may be necessary to test the reactivity of this reagent against sera of humans or animals infected with closely related viruses such as HSV-1, HSV-2, or SA8 to better assess specificity. The low sensitivity of the assay at hand for sera that were previously established to have a titer of less than 5000 was most likely a result of decreased antigen-antibody interaction because of the low concentration of total antibodies in these sera, and subsequently lower concentration of antibodies specific to glycoprotein D. This is also consistent with reports that gD antibodies usually appear later than gB antibodies during the course of natural infection and are of lower titers (Ashley, Benedetti et al. 1985) (Eberle, Mou et al. 1985). It is also possible to speculate that the decreased sensitivity of the assay for these sera was due to a diminished presence of antibodies specific to structural glycoproteins, or alternatively due to an altered conformational structure in the purified recombinant gD 287 that decreased the functional affinity of antibodies directed to the native form.

In regards to the first speculation, the minimal presence of antibodies to structural glycoproteins in favor of more pronounced presence of antibodies directed towards low molecular weight proteins of the nucleocapsid in sera from patients with primary HSV-1 infections has been reported (Eberle, Mou et al. 1985). This observation led to speculation that during primary infection in the macaque, B virus induced antibodies with specificity for low molecular weight virus or virus-induced proteins (mainly 39,000 to 50,000 kDa). It is important to note, however, that Eberle et al. used western blot in the above study, and therefore their results reflect an evaluation of antibodies that react with membrane-bound denatured antigens, and may not be applied to explain the low sensitivity with our recombinant in the context of ELISA. Moreover, early responses against glycoproteins including glycoprotein D have been documented in HSV-1 and HSV-2 infected patients using sequential serum by immunoprecipitation followed by gel electrophoresis (RIPA-PAGE) (Ashley, Benedetti et al. 1985). The question of whether sera collected during certain stages of infection contained a prevalent response against glycoproteins was undertaken for HSV-1 and HSV-2 by a comprehensive study that used both western blot and RIPA-PAGE (Kuhn, Dunkler et al. 1987). Using methods of IgM and IgG separation, and more stringent controls (titer adjustment, VZV gB cross-reactivity exclusion), the investigators documented reactivity to glycoproteins (including gD) directly after infection, during acute infection, and in seropositive individuals with no clinical symptoms. IgM reactivity to glycoproteins was scarcely detected in early and recurrent infections in either assay. Other reports noted the early appearance of antibodies to structural glycoproteins especially in genital infections; the glycoprotein to which an antibody response was detected varied based on the type of infecting virus (HSV-1 or

HSV-2), the anatomical site of infection (oral vs genital) and the history of exposure (primary or recurrent) with reactions to glycoprotein B and glycoprotein D significantly increased by labial recurrences (Kahlon, Lakeman et al. 1986; Bernstein, Frenkel et al. 1990). In the guinea pig model, recurrences were inversely related to gD antibody titer in the genital HSV infections (Bernstein, Stanberry et al. 1987). Although some of these results seem contradictory, the use of antigen in these reports varies from native to denatured and soluble to solid-phase-bound. The sera used come from different stages of infection, many times not well defined due to limited availability of clinical background information. It is worth noting also that the avidity of antibodies increases with time after exposure, be it primary exposure or recurrent infection (Werblin, Kim et al. 1973; Hashido, Inouye et al. 1997); taken together with assay specific-limitations, some specific antibody responses may be missed because the antigen-antibody interaction is not stable enough under assay conditions. In the current study, clinical information relevant to time of infection was not available on the animals tested and it was not possible to directly relate the antibody titers to certain stages of disease development. In addition, at this time it is not possible to distinguish between primary and recurrent B virus infections reliably. It is also unlikely that each sample in our hands was collected from macaques with primary (or recurrent) infections since sera were picked randomly. An interesting future quest would be to determine any correlation between the stage of disease development and the antibody profile in B virus infections under controlled conditions. The serological antibody profile to B virus is not completely defined and is complicated by factors of cross-reactivity resulting from previous infections with closely related viruses, the stage of infection, and host specific responses.

The relatively low optical density which was associated with sera previously shown to have lower titers (<5000) might have been related to reduced affinity of antibodies that recognized gD 287. Altered folding of gD due to inauthentic glycosylation, chemical conditions, or truncation could have also affected the antigenic valence, i.e. a decrease in the number of epitopes on the surface of gD287 available to effectively bind antibody paratopes. This would have resulted in reduced binding activity as the stability of binding is adversely affected by fewer interactions. In comparison to mammalian cells, the altered glycosylation, processing, and packaging mechanisms in insect cells could have contributed to a final conformation of gD287 that was antigenically somehow different from native gD, and thus had lower affinity to anti-gD antibodies in the tested sera. This could be the case especially if binding was altered by specific sugar moieties. It is known that N-Glycan structures produced in the baculovirus system are less complex than those presented in mammalian cells, since insect cells have no detectable sialyltransferase activity and lack enzymes for N-glycan elongation (Altman, Staudacher et al. 1999; Marchal, Jarvis et al. 2001). We know from biopharmaceuticals production research that the recombinants lacking sialylation had a shorter half-life in general. Although most enzymes produced in baculovirus systems retained their biological function, the possibility of less stable antigen-antibody interaction as a result of altered glycosylation cannot be completely excluded (Luckow and Summers 1988; O'Reilly 1997; Jarvis, Howe et al. 2001).

Another possibility is that truncation *per se* caused conformational changes that impinged on antigenicity of glycoprotein D. In that context, the structure of B virus gD

has not been elucidated but that of its homolog HSV-1 gD has been well studied. It has been shown that significant conformational changes in HSV-1 gD take place as it binds the different receptors that mediate virus entry. The N-terminus of gD adopts either an extended and flexible conformation when it interacts with nectin-1 or a hairpin structure when it interacts with HveA (Carfi, Willis et al. 2001; Connolly, Landsburg et al. 2005). Prior to gD interacting with its receptors, the extracellular C-terminus of gD beyond residue 260 is believed to wrap around the core of gD and conceal receptor-binding sites in free virions. This section of the C terminus moves during receptor binding and this movement is thought to play a role in bringing the viral membrane to proximity with the cell membrane in preparation for virion membrane fusion. Glycoprotein D is also expressed on the cell surface of infected cells and shown to play a role in cell-cell spread of the virus (Johnson and Smiley 1985; Srinivas, Balachandran et al. 1986; Dingwell, Brunetti et al. 1994). Thus, the presentation of gD to the immune system varies with the progress of the infection and the type of tissue that is infected. One can speculate as to whether the sera with low total titer had antibodies specific to epitopes of gD that are absent or cryptic on the recombinant gD we used. The antibody specificities in sera of B virus infected animals have not been fully elucidated but the prevalence of such specificities during HSV infection and their relation to infection stages suggest a similar pattern in B virus. Previous studies in our lab indicated that a number of positive macaque sera contained antibodies directed to denaturation-sensitive epitopes of the ectodomain of B virus glycoprotein D (Perelygina, Zurkuhlen et al. 2002). These sera were not reactive to an immunodominant linear epitope on the cytoplasmic tail of gD, gD 362-370. It would be useful to test the reactivity of these specific sera (where available)

to gD 287 to determine reactivity of gD 287 to antibody paratopes directed against conformational epitopes of the ectodomain. Whether this difference in antibody profiles is host specific or related to the strain of infecting virus is another interesting question. The ideal antigen to detect antibodies against B virus gD may be a combination of immunodominant linear epitopes (e.g gD 362-370) and native ectodomain of gD (e.g gD 287). In addition, a conclusive diagnostic assay will most likely involve an array of glycoproteins, including gC, gB, gL, gI, and gH. Standardization of such an assay and optimization of its diagnostic value is definitely a priority of B virus diagnosis research besides the added value these reagents present in bioassays that explore their role as receptors in virus entry, spread, and immune evasion. In this context, recombinant gD may be important in exploring the role of gD/nectin-1 interaction and its possible role in cell-cell spread, especially in the CNS where B virus ability to cross the trigeminal ganglia in humans may be one of the most important reasons for its lethality. Glycosylation deficiencies of the baculovirus system may hamper the reagents' bioactivity although this has not been the case in studies with HSV-1 gD interaction with HVEM where a gD mutant in which all N-glycosylation sites were ablated still bound HVEM in a specific manner (Whitbeck, Peng et al. 1997).

It is also imperative to test gD 287 against a larger sample of high titer positive sera in both macaques and humans to establish sensitivity at a higher confidence interval. The study at hand represented more of a pilot study than one whose goal is the establishment of diagnostic value guidelines, although repeated testing of gD 287 with other positive sera is beneficial when taken in the context of reactivity of other recombinant glycoproteins (pers. comm. , Katz D., 2007).

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