Universidade Estadual de Londrina

NEWTON HASHIMOTO

DESENVOLVIMENTO E PADRONIZAÇÃO DE MÉTODO IMUNOENZIMÁTICO PARA A DETECÇÃO DE ANTÍGENOS DE POLIOVÍRUS 1 E HERPESVÍRUS BOVINO 1

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Dissertação apresentada como parte dos requisitos para a obtenção do título de Mestre em Microbiologia da Universidade Estadual de Londrina

Orientador: Prof. Carlos M. Nozawa

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1 INTRODUÇÃO

O diagnóstico laboratorial das viroses envolve a aplicação de métodos de identificação de patógenos para a predição do curso da infecção e o estabelecimento de um procedimento terapêutico apropriado. O diagnóstico laboratorial baseia-se na demonstração direta ou indireta do patógeno ou de seus produtos nos tecidos, secreções e excreções. Esta informação pode ser gerada através de exame microscópico, cultivo e identificação de vírus, avaliação da resposta imune do hospedeiro e detecção de macromoléculas patógeno-específicas nas amostras clínicas (Santos, 2002; Engleberg, 1999).

O isolamento de vírus é o método tradicional da virologia e consiste no cultivo do agente em um hospedeiro suscetível, que compreendem, animais, ovos embrionados e cultivo de células (Candeias, 1998). Uma vez inoculados nos hospedeiros, os vírus provocam modificações fisiológicas nestes sistemas biológicos, como conseqüência da replicação viral nas células competentes. Os fenômenos de alteração morfológica (efeito citopático), hemaglutinação, hemadsorção, paralisia, morte ou lesões degenerativas são exemplos dos efeitos decorrentes da infecção viral. Contudo, estas alterações apenas evidenciam a replicação viral, necessitando da utilização de testes sorológicos, como o teste de neutralização, teste de fixação de complemento ou teste de inibição da hemaglutinação que empregam soros padrões para a identificação do vírus (Santos, 2002).

A observação direta de vírus nas amostras clínicas através da microscopia eletrônica constitui um método simples e rápido. A microscopia

eletrônica permite a observação da morfologia das partículas virais, por contraste, com substância de alta densidade aos elétrons (Miller, 1995).

A sorologia desempenha um importante papel no diagnóstico clínico e na investigação epidemiológica dos vírus. Os testes sorológicos são úteis na determinação de uma infecção aguda, do estado imunológico do paciente e no estudo epidemiológico do comportamento de uma virose numa comunidade. Estes testes avaliam a resposta humoral de um indivíduo para uma infecção.

Nos últimos anos, as técnicas para a detecção e quantificação de vírus, através de ácido nucléico viral, tornaram-se um valioso instrumento nos laboratórios clínicos e de pesquisa na virologia. Estas técnicas compreendem, por exemplo, a PCR (polymerase chain reaction), bDNA (branched chain signal amplification), NASBA (nucleic acid sequence-based amplification), SHARP signal and hybrid capture systems e as técnicas de hibridação molecular (Dot blot, Southern blot e Northern blot) (Hodinka, 1998). Sua importância reside nas situações em que os métodos convencionais não são apropriados, como na identificação de vírus não cultiváveis, infecções com produção limitada de antígenos (latência) ou nos resultados sorológicos duvidosos (Garson, 1994). Além disso, a aplicação dessas técnicas pode melhorar a habilidade de predizer a progressão de uma doença, a eficácia do procedimento terapêutico de drogas e vacinas, e monitorar o desenvolvimento de resistência a agentes antivirais (Hodinka, 1998; Clewley, 1989). Apesar destes benefícios, estas técnicas apresentam ainda várias limitações. Estas técnicas são relativamente complexas, demoradas e de elevado custo (Garson, 1994). A extrema sensibilidade exacerba o problema de falso positivo devida à contaminação das amostras (Garson, 1994). Além disso, o genoma viral pode sofrer

degradação se as amostras não forem imediatamente armazenadas em condições ideais, resultando em falso negativo (Garson, 1994).

O poliovírus é um membro da família *Picornaviridae*, gênero *Enterovirus*, causador da poliomielite, uma doença infecciosa aguda que, na sua forma mais grave, leva à paralisia flácida ou até à morte por parada cardíaca ou respiratória. O vírion consiste de um capsídio (27-30 nm de diâmetro) com 60 subunidades, cada qual com quatro proteínas (VP1-VP4), dispostas em simetria icosaédrica em torno de um genoma constituído por um único filamento simples de RNA de sentido positivo (Melnick e Butel, 2000b).

A incidência de pólio no mundo diminuiu marcadamente desde a disponibilidade das vacinas (Eggers, 1999). Após 15 anos de esforços, com envolvimento de mais de 200 países, 20 milhões de voluntários e um investimento de US$ 3 bilhões, o mundo encontra-se próximo de tornar-se livre da poliomielite (WHO, 2004). A doença é endêmica na Nigéria, Índia, Paquistão, Afeganistão, Niger e Egito. Os programas epidemiológicos são importantes para avaliar a eficiência das campanhas de vacinação e demostrar a erradicação do vírus.

Um dos instrumentos utilizados neste intento consiste no exame da cepa de poliovírus isolado de casos clínicos e o método aplicado deve ser sensível e capaz de processar um número grande de amostras clínicas e ambientais (Minor, 2000).

Na prática, o estudo do poliovírus é baseado, principalmente, na avaliação sorológica dos níveis de anticorpos e identificação de isolados através do teste de neutralização (Payment et al., 1982; Wahby, 2000). Este teste é sensível e específico. Contudo, por utilizar cultura de células, apresenta algumas desvantagens como elevado custo e demora na obtenção de resultados. Além de que a

interpretação dos títulos de neutralização pode ser influenciada pela análise do efeito citopático. Outros métodos de identificação consistem no isolamento do vírus (Lennette, 1995) e no uso da microscopia eletrônica, imunofluorescência e imunoeletroforese (Payment et al., 1982). Estas técnicas, entretanto, não são justificadas para o processamento de grande número de amostras (Payment et al., 1982). A microscopia eletrônica é um método rápido e simples, mas seu uso é restrito pelo alto custo do equipamento, limite de detecção baixo (10^6 partículas ml^{-1}), processamento de um número limitado de amostras e análise subjetiva do investigador (Czerny e Eichhorn, 1989). Os ensaios de imunofluorescência são rápidos e sensíveis mas podem apresentar dificuldades quando houver excesso de fluorescência inespecífica.

O herpesvírus bovino 1 (BHV-1) é um membro da família *Herpesviridae*, subfamília *Alphaherpesvirinae*, gênero *Varicellovirus* (Babiuk et al., 1996). O vírion apresenta um diâmetro de 120-200 nm, possui um genoma de DNA de duplo filamento, linear, encerrado em quatro camadas: um cerne interno circundado por um capsídio icosaédrico, seguido de um tegumento amorfo e um envelope lipídico. (Rixon, 1993; Melnick e Butel, 2000a; Engels e Ackermann, 1996).

O BHV-1 é o agente causador de uma série de enfermidades, incluindo a rinotraqueíte infecciosa, vulvovaginite infecciosa, conjuntivite, balanopostites e abortos (Hanon et al., 1999; Babiuk et al., 1996; Teixeira et al., 2001). O BHV-1 tem distribuição ampla, estando presente em quase todos os países de bovinocultura expressiva. Em alguns países da Europa, a infecção já encontra-se em vias de erradicação. Sua importância econômica é tão marcante que a enfermidade deverá ser a próxima barreira sanitária ao comércio internacional de bovinos e correlatos na União Européia. Os levantamentos sorológicos realizados no

Brasil têm demonstrado alta freqüência de rebanhos soropositivos com índices que variam de 10.7% a 85.7% (Richtzenhain et al., 1998). Ainda no Brasil, um levantamento sorológico, realizado em 2.447 amostras de soro bovino provenientes de 56 rebanhos distribuídos pelos estados do Paraná, São Paulo, Minas Gerais, Rio de Janeiro, Mato Grosso do Sul e Rio Grande do Sul, encontrou uma freqüência de 69% (1.681/2.447) de animais soropositivos (Richtzenhain et al., 1998). Portanto, o Brasil possui grande parte das propriedades com animais sorologicamente positivos para o BHV-1, o que salienta a importância de adoção de medidas com vistas a identificar estes animais, os quais são potenciais disseminadores da infecção (Teixeira et al., 2001).

O diagnóstico da infecção por BHV-1 baseia-se nos sinais clínicos característicos confirmados por exames laboratoriais. Os testes laboratoriais, tradicionalmente usados, consistem no isolamento do vírus em cultura de células (Collins et al., 1988), sorologia pareada e na detecção de antígenos virais por imunofluorescência em cortes histológicos coletados *post mortem* e em esfregaços de células do epitélio nasal e vaginal (Edwards e Gitao, 1987). O isolamento do vírus é um ensaio demorado, dispendioso, principalmente, para grande número de amostras e títulos virais baixos, e exige experiência técnica (Collins et al., 1988). A chance de isolamento do vírus diminui com a duração da doença e as amostras de campo devem ser preservadas durante o transporte para o laboratório para evitar a perda da infecciosidade (Kok et al., 1998; Collins et al., 1988). A sorologia pareada é uma técnica demorada e dispendiosa.

A detecção de antígenos virais em amostras clínicas é uma alternativa rápida e econômica. A técnica de imunofluorescência em cortes histológicos e esfregaços de células nasais é amplamente usada no diagnóstico de BHV-1. Ensaios imunoenzimáticos têm sido descritos na detecção de antígenos no muco nasal e nas células infectadas, oferecendo várias vantagens, que incluem, a rapidez, simplicidade e a obtenção de resultados objetivos (Middeldorp et al., 1987; Gleaves et al., 1990).

2 HISTÓRICO DO ENSAIO IMUNOENZIMÁTICO (EIE)

O surgimento dos imunoensaios modernos pode ser atribuído ao estabelecimento de marcadores capazes de demonstrar a interação antígeno-anticorpo.

Yallow e Berson (1960) desenvolveram técnicas de marcação com radioisótopos para a determinação do nível de complexos insulina-anti-insulina em diabetes tipo 1. Este trabalho formou a base do radioimunoensaio (RIA). A sensibilidade e aplicabilidade do RIA, na quantificação de compostos de baixo peso molecular, permitiu sua rápida expansão na rotina dos laboratórios clínicos e de pesquisa (Deshpande, 1996a; Sanchez, 2001; van Weemen, 1985).

Singer e Schick (1960) usaram um derivado de isocianato na conjugação da ferritina com anticorpos. Subseqüentemente, Avrameas e Uriel (1966); Nakane e Pierce (1966) demostraram a conjugação de enzimas com anticorpos.

Catt e Tregear (1967) descreveram que tubos de poliestireno possuíam a propriedade de adsorver proteínas, irreversivelmente, e que anticorpos adsorvidos conservavam sua avidez pelo seu antígeno.

Estas descobertas permitiram a introdução de marcadores enzimáticos por Engvall e Perlmann (1971); van Weemen e Schuurs (1971) para os imunoensaios. O termo ELISA (Enzyme linked immunosorbent assay) foi introduzido por Engvall e Perlmann em 1971 para descrever um ensaio imunoenzimático a qual um dos reagentes está adsorvido numa superfície sólida. O ELISA foi desenvolvido como alternativa para o RIA, apresentando a mesma sensibilidade sem a necessidade de reagentes radioativos (Deshpande, 1996a; Sanchez, 2001; van Weemen, 1985).

3 CARACTERÍSTICAS DO ENSAIO IMUNOENZIMÁTICO

As características mais importantes do EIE são a sensibilidade, a especificidade e a praticidade (van Weemen, 1985; Porstmann e Kiessig, 1992). O método é simples e leva apenas 3-4 horas para a obtenção de resultados (Grandien, 1996). O seu limite de detecção alcança níveis da ordem de nanograma de proteínas. Por exemplo, na detecção do antígeno de superfície do vírus da hepatite B (HBsAg), o limite de detecção da técnica de imunoprecipitação é da ordem de 100ng/ml, enquanto que o EIE é de 1 ng/ml (Pillot, 1996). Esta característica deve-se à capacidade do imunoensaio de medir diretamente a interação de antígeno e anticorpo, não dependendo de um segundo fenômeno, como precipitação, aglutinação ou fixação de complemento. Este segundo fenômeno requer a formação de complexos de antígeno-anticorpo maiores, diminuindo a sensibilidade do teste (Deshpande, 1996b; Sanchez, 2001). Além disso, o uso de enzimas potencializa o sinal, na fase de detecção, da interação antígeno-anticorpo, pois uma única enzima é capaz de converter um grande número de moléculas de substrato (Grandien, 1996).

Atualmente, existem vários recursos que permitem o aumento da sensibilidade dos ensaios imunoenzimáticos.

O sistema (strepto)avidina-biotina aumenta a relação molar de enzima:antígeno enquanto as reações enzimáticas cíclicas aumentam o sinal final da reação (Jackman, 1986; Grandien, 1996).

A avidina é uma glicoproteína que possui quatro sítios para a vitamina biotina (Deshpande, 1996c). No sistema avidina-biotina, uma molécula de anticorpo é marcado com 10-20 moléculas de biotina (Deshpande, 1996c). Cada

molécula de biotina é reconhecida por uma molécula de avidina conjugada ou não com enzima (Deshpande, 1996c). Este sistema apresenta três configurações diferentes: LAB (labeled avidin-biotin), BRAB (bridged avidin-biotin) e ABC (avidin-biotin complex) (Deshpande, 1996c). No método LAB, a biotina associada ao anticorpo liga-se com a avidina marcada com enzima (Deshpande, 1996c). No método BRAB, a biotina do anticorpo liga-se a avidina, que, posteriormente, estabelecerá ligação com uma ou mais moléculas de biotina conjugada com enzima (Deshpande, 1996c). No método ABC, a avidina liga-se a biotina marcada com enzima de modo a formar um polímero desta duas moléculas(Deshpande, 1996c).

As reações enzimáticas cíclicas aumentam o sinal da reação pelo uso de enzimas que produzem um catalizador para uma segunda reação ou uma série de reações. O exemplo mais conhecido consiste da reação da fosfatase alcalina na reciclagem do cofator NAD, que catalisa a reação de oxido-redução para dois sistemas enzimáticos usados simultaneamente (Jackman, 1986).

O uso de anticorpos monoclonais permitiu o aumento da sensibilidade e especificidade devido à seleção de anticorpos com alta afinidade a um determinado epítopo no antígeno analisado (van Weemen, 1985).

Atualmente, as diferentes técnica imunoenzimáticas são as mais utilizadas para detecção de antígenos e anticorpos devido à capacidade de avaliar várias amostras simultaneamente, em um tempo relativamente curto, com a máxima sensibilidade e o mínimo de experiência técnica e equipamentos (Pillot, 1996).

4 OBJETIVOS

4.1 GERAL: Desenvolvimento de teste imunoenzimático para monitoramento de antígenos virais.

4.2 ESPECÍFICO: Monitorar a replicação de poliovírus e herpesvírus bovino em culturas de células, nos experimentos de antivirais, e também para a detecção dos respectivos vírus em materiais de campo, para fins diagnóstico.

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ARTIGO 1: Development of enzyme immunoassay for detection of Bovine Herpesvirus 1 (BHV-1) antigens

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ABSTRACTS

An indirect solid-phase enzyme-immunoassay (EIA) was developed for the detection of bovine herpes virus 1 (BHV-1) antigen. BHV-1 antigen was obtained in HEp-2 cell culture and used to immunize rabbits and guinea pigs. The BHV-1 antibodies were evaluated by double immunodifusion in agar gel and neutralization test. Optimal concentrations of guinea pig and rabbit immunoglobulins were determined by checkerboard titration. Microtitre plates were coated with 9.0 ug/ml guinea pig anti-BHV-1 immunoglobulin and rabbit anti-BHV-1 immunoglobulin at the concentration of 15.0 ug/ml was used as detecting antibody. The performance of EIA was analysed from standard curve by measuring the coefficient of variation (CV) and the establishment of dose response relationship. The standard curve with eight different concentrations in five replicates resulted in a CV between 3.14% to 15.76%. The dose response relationship was determined by simple linear regression (OD = 0,0149 x [Ag] + 0,4041) with a coefficient of correlation, R^2, equal to 96.98%. The assay detected a minimum of 1.6 ug/ml BHV-1 antigen.

INTRODUCTION

The approaches for laboratory diagnosis of virus infection have been the isolation of the agent in host systems and/or detection and measurement of antibody in host serum (Santos, 2002; Lennette, 1995; Engleberg, 1999). The development of virus infection laboratory diagnosis has been toward rapid methods capable to provide answer in less than 24 hours. The best methods should fulfil the five prerequisites of speed, simplicity, sensitivity, specificity and low cost. Tests for viral antigen, which provide a diagnosis from a single specimen, are rather important for many purposes. Solid-phase enzyme immunoassay (EIA) is one of the methods of choice.

Bovine herpesvirus type 1 (BHV-1), a member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus*, is a major cause of economic loss to livestock producers (Hanon et al., 1999; Teixeira et al., 2001). It causes infectious bovine rhinotracheitis, genital infections leading to abortions and fetal death (Babiuk et al., 1996; Engels and Ackermann, 1996).

The specific diagnosis of BHV-1 has traditionally been based on the isolation of causal virus in cell culture, together with the detection of an active antibody response in the host animal (Edwards and Gitao, 1987). The latter method takes two weeks because of interval required between the two samples.

Virus isolation can be hampered by several factors, such as, time consuming, cost and technical experience (Collins et al., 1988). The chance for virus isolation decreases with the duration of illness and the specimens must be preserved during transport to laboratory because of the loss of virus infectivity, cause of false negative results (Kok et al., 1998; Collins et al., 1988). The observation for specific cytopathic changes (CPE) in inoculated cultures is labour intensive, particularly, for large

number of samples and long time required for low virus titers to develop CPE (Kok et al., 1998; Payment et al., 1982).

The detection of viral antigen in clinical samples can be a rapid and economical alternative to cell culture. The most widely applied antigen detection technique has been immunofluorescence (IF), either on smears of cells from the nasal or ocular epithelium or on tissues sections collected at post mortem. EIA has been described for labeling of infected cells and for the detection of soluble antigen in nasal mucus. None of these two antigen detection methods for BHV-1 attained the sensitivity of virus isolation in cell culture (Edwards and Gitao, 1987).

EIA offers several advantages including speed, ease of performance and objective results (Deshpande, 1996a; Middeldorp et al., 1987; Gleaves et al., 1990). It is the most widely used of all immunological assay for antibodies and antigens, since large numbers of test can be performed in relative short time with maximum of sensitive and minimum technical expertise and equipment (Pillot, 1996; van Weemen, 1985).

This paper describes an EIA for the detection of BHV-1 antigens.

2.MATERIALS AND METHODS

VIRUS AND CELL CULTURE

BHV-1, supplied by Laboratório de Virologia (DMVP/UEL), was grown in HEp-2 cell cultures. The cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Chem. Co, USA.) supplemented with 7% fetal bovine serum (Gibco BRL, USA), 100 ug/ml streptomycin (Sigma Chem. Co, USA), 100 UI/ml penicillin (Sigma Chem. Co., USA) and 2.5 ug/ml fungizon (Bristol-Myers Squibb, Brazil).

ANTIGEN PRODUCTION AND PURIFICATION

The supernatant fluid of virus-infected cell cultures was harvested when cultures showed maximal CPE, usually 24-48 hours post-inoculation. The virus titer was determined by $TCID_{50}$ as 0.5 x 10^7/ml. The supernatant fluid of infected cell cultures was clarified by centrifugation at 10,000 x g for 30 min. Partial concentration of virus was carried out by the addition of polyethylene glycol (PEG) 6000 (8%, W/V) at 4^oC overnight under agitation and followed by centrifugation at 10,000 x g for 30 min at 4^oC. The resulting precipitate was resuspended in 10 ml PBS, pH 7.4, and 1.0-2.0 ml layered on a cushion of 20% sucrose in PBS, and ultracentrifuged in a Beckman SW41 rotor at 100,000 x g for 3 hours. The pellet was resuspended in 4 ml PBS. The protein content was determined by the method of Folin-Ciocalteau, and the material was stored at -20^oC.

IMMUNIZATION OF ANIMALS

Rabbits and guinea pigs were inoculated five times at intervals of one week with 0.2 ml purified viral antigen suspension emulsified with an equal volume of Freund's Complete Adjuvant. The animals were bled by cardiac puncture seven days after the final booster.

ABSORPTION OF SERA

Viral antisera free from HEp-2 cell antibodies were prepared by absorbing them with 10^6 cells/ml suspension (vol/vol), maintained overnight under constant agitation at 12^oC followed by centrifugation at 1,000 x g for 10 min (Tyrrell, 1973).

DETERMINATION OF ANTIBODY TITERS

Double immunodiffusion in agar gel was used for screening poolled antibodies raised in rabbit and guinea pig against viral antigens, and was performed as previously described (Johnson, 1986). Neutralization test was carried out as follows. Test sera were prepared in twofold dilution in cell culture maintenance medium. Thereafter, 100 ul volume of each dilution was mixed with an equal volume of 100 $TCID_{50}$ of virus and incubated at 37°C for 1 hour. The mixture was inoculated onto cell monolayers grown in 96-well tissue culture plates and incubated at 37°C for 5 days in 5% CO_2 atmosphere. The antibody endpoint (titer) was determined as the highest dilution of the serum that inhibits CPE.

PRECIPITATION OF IMMUNOGLOBULINS

A volume of hyperimmune serum pool was diluted with same volume of distilled water. Total immunoglobulin was precipitated by slowly addition of equal volume of ammonium sulfate 50% saturated followed by centrifugation at 1,000 x g for 10 min. The precipitate was dissolved in PBS and the precipitation process repeated twice. The final precipitate was dissolved in approximately 4 ml PBS and dialyzed overnight against PBS at 4°C. The protein content was determined by the method of Folin-Ciocalteau, and the material was stored at -20°C.

INDIRECT ENZYME IMMUNOASSAY PROCEDURE FOR CHECKERBOARD TITRATION

The procedure for the detection of viral antigens was established using an antigen capture EIA similar to that described by Ziegler et al. (1983). Optimal concentrations of guinea pig and rabbit immunoglobulins were determined by checkerboard titration

on microtiter plates. Typically, protein concentration of 1 ug/ml is used to saturate the surface (Deshpande, 1996b); and should not exceed 10 ug/ml IgG for solid-phase adsorption (Porstmann and Kiessig, 1992). Thus, the guinea pig antibody concentrations were estimated as the established limit above. Briefly, flat bottom polystyrene microtiter plates (Hemobag, Brazil) were coated with 100 ul per well of guinea pig antibodies diluted at 0.9 ug/ml (half plate) and 9.0 ug/ml (half plate) in 0.1 M carbonate-bicarbonate buffer, pH 9.6, overnight, at 4°C, in a humidified chamber. Plates were washed twice with PBS, pH 7.2, containing 0.05% TWEEN 20 (PBS-T). The coated plates were blocked with 200 ul/well of blocking solution (PBS, pH 7.2, containing 2% skimmed milk powder and 0.05% TWEEN 20) for 90 min at 37°C. Plates were washed thrice with PBS-T throughout, unless otherwise indicated, and 100 ul/well in one column with PBS (blank), two columns for each viral antigen concentration at 2.0 and 20.0 ug/ml as positive control were incubated at 37°C for 60 min. Plates were washed with PBS-T. Aliquots of 100 ul of rabbit antibody, diluted in a serial dilution from 60.0 ug/ml to 1.9 ug/ml, in blocking solution, pH 7.2, were added and the plates incubated at 37°C for 60 min. Incubation was followed by washing and the addition of 100 ul of goat anti-rabbit IgG peroxidase-conjugate (Sigma Chem. Co., USA) at 1:40,000 in blocking solution, pH 7.2, for 60 min at 37°C. Plates were washed and followed by the addition of 100 ul of substrate (0.03% H_2O_2) and chromogen, 400 ug/ml *o*-phenylenediamine dihydrochloride (Sigma Chem. Co., USA) diluted in 0.05 M citrate-phosphate buffer, pH 5.0. After 15 min at 25°C in light tight box, the reaction was stopped by the addition of 50 ul 2 N H_2SO_4 and the optic density (OD) read at 490 nm.

ANTIGEN STANDARD CURVE FOR DOSE RESPONSE

The standard curve was generated from measures of decreasing amounts of antigen concentration (200.0 ug/ml to 1.6 ug/ml) in five replicates by previous protocol with the immunoglobulin concentrations optimized by checkerboard titration (9.0 ug/ml for capture antibody and 15.0 ug/ml for detector antibody).

STATISTICAL ANALYSIS OF DATA

The standard curve was used to estimate the dose-response relationship and the variation associated with the measurement of the analyte concentration.

The coefficient of variation, CV = (SD/mean) x 100, of each point of the standard curve was estimated by assaying this point five times.

The dose response relationship was determined by linear regression to determine whether two or more variables are linearly related (Microsoft EXCEL).

The selection of data points for a given dilution curve was empirical. The analysis was based on the number of data points and the correlation coefficient (R^2). Normally, at least four data points are included and a R^2 equal or larger than 95%.

CLINICAL SAMPLES

One negative bovine vaginal swab and three BHV-1 positive swabs (one nasal and two vaginal), in 1.0 ml PBS, were provided by the Hospital Veterinário/UEL. These specimens were assayed by cell culture virus isolation. Eight replicates of each sample diluted at 1:10 were tested by the previous optimized condition.

CUT-OFF

The cut-off value is defined as mean negative + 2.8 x standard deviation of negative specimens with a confidential interval of 99.8%, according to Porstmann and Kiessig (1992).

3.RESULTS

The presence of anti-BHV-1 immunoglobulins in guinea pig and rabbit sera was determined by double immunodiffusion in agar gel and neutralization test. The pattern of precipitin lines in double immunodiffusion test indicated a strong reaction against the purified BHV-1 antigens, on day 28 after the first injection, for both rabbit and guinea pig hyperimmune sera. A moderate response against cellular antigens was also observed. These hyperimmune sera evaluated by neutralization test demonstrated titers of 1: 2,560 and 1: 1,280 for guinea pig and rabbit sera, respectively.

The determination of protein concentration of cellular and viral antigens and immunoglobulins was carried out to proceed checkerboard titration and standard curve analysis.

The concentrations of protein in HEp-2 and purified BHV-1 antigen preparations were estimated in 7.7 mg/ml and 2.0 mg/ml, respectively, and for antisera in 9.0 mg/ml and 0.6 mg/ml, respectively, for guinea pig and rabbit.

Optimal concentrations of capture and detector immunoglobulins were determined by checkerboard titration against the antigen used for immunization (Schoenthaler and Kapil, 1999; Deshpande, 1996b; Voller and Bidwell, 1986). Each combination of

capture and detector antibodies was tested in the presence of negative, 2.0 ug/ml and 20.0 ug/ml of antigen samples. The optimum immunoglobulins concentrations were those giving the greastest and most reproducible changes in signal with increasing antigen concentration. The initial analysis indicated high background due to an excessive non-specific binding (NSB) hampering distinction of a positive signal derived from the analyte against the blank values. Increasing blocking agent concentration from 1% to 2%, we minimized this type of NSB. Increasing the number of washes also alleviated this problem to a large degree. The concentration of guinea pig antibody (capture) of 9.0 ug/ml demonstrated a better result than 0.9 ug/ml. The slope of the curve indicated that suitable results would be achieved by the use of 9.0 ug/ml of capture antibody and 15.0 ug/ml of rabbit antibody (detector).

By the standard curve, we could evaluate the precision and the quantitative aspect of the assay. The precision, also referred as reproducibility, is a statistical measure of variation between repeated determination of the same sample. The statistical index used to express the precision profile is the coefficient of variation, calculated as following, CV = (Standard Deviation/Mean) x 100 at a particular antigen level. Different concentrations of antigen were used to evaluate the performance of the method on the same day in five replicates and the CV varied from 3.14 to 15.76 %.

When evaluating the dose response by standard curve for decreasing concentration of reference antigens, a sigmoid curve was obtained. Simple linear regression was used to determine if the assay showed a statistically significant dose response relationship in which the OD was directly proportional to the antigen concentrations (Wilbur, 1993). Antigen concentrations chosen empirically was analyzed to reach the best correlation (R^2) between OD and antigen concentration. The linear interval occurred between 1.6 ug/ml and 25.0 ug/ml and resulted in a dose response

relationship represented by OD = 0,0149 x [Ag] + 0,4041, with the coefficient of correlation, R^2, equal to 96.98%.

In the optimized condition, our assay detected cell antigen (HEp-2) only at levels higher than 770 ug/ml indicating that the assay had a low level of cross reaction with cell antigen.

Our assay detected a minimum of 1.6 ug/ml BHV-1 antigen.

The positive clinical samples resulted in OD of 0.271, 0.300, and 0.179, while the cut-off value resulted in an OD of 0.202.

4.DISCUSSION

We developed an adaptation of an EIA for the detection of BHV-1 antigen.

Specific antibodies for BHV-1 antigens were demonstrated by double immunodifusion in agar gel and titered by neutralization tests suggesting the efficacy of viral purification and immunization procedures.

Optimal concentrations of rabbit and guinea pig immunoglobulin were evaluated by checkerboard titration and the concentrations selected on the basis of maximal sensitivity and minimum non-specific reactions.

The solid phase has a limited capacity for binding proteins. Most microtiters plates are capable of binding approximately 0.4 pg/cm^2, and when antibody concentration exceeds 1 ug/cm^2, unstable bi- and polylayers are formed, from which analyte molecules binding to second layers are removed as antigen-antibody complexes by bound-free separation (Desphande, 1996b; Porstmann and Kiessig, 1992). Thus, we preferred to test first the minimum concentration of capture antibody that allowed high

sensitivity without exceeding the immunosorbent capacity of solid phase. High concentrations of detector antibody have also a disadvantage of the increased background that offsets the benefits of the signal magnitude.

Precision is probably the most important technical aspect of an assay performance. The performance of the assay was demonstrated to be good by determining the CV. This was over the measurement range of EIA of < 15%, whereas 30% is the maximum acceptable value for repeatability of a diagnostic test (El Idrissi and Ward, 1992; Porstmann and Kiessig, 1992; Deshpande, 1996b; Gubbels et al., 2000). Our assay demonstrated a CV varying from 3.14 to 15.76 %.

Our assay detected a minimum of 1.6 ug/ml BHV-1 antigen. Most of EIAs detect antigen at level of 1 ng/ml (Deshpande, 1996b). According to our data, it is suggested that there is still a significant background or the avidity of antibody is not sufficient to detect lower levels of the antigens. Improving the final detection system with, such as, avidin-biotin or coupled enzyme cascade systems could solve it (Grandien, 1996; Jackman, 1986).

One of the field specimens resulted in an OD 0.179 bellow the cut off OD 0.202. Therefore, considered false negative, and this event in discussed by Gleaves et al. (1990).

In conclusion, we described an antigen detection test for HBV-1 antigens, which has a potential use in diagnostic procedures, and also for monitoring the antiviral properties of substances in the replication of the virus.

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ARTIGO 2: Development of enzyme immunoassay for detection of poliovirus 1 antigens

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ABSTRACTS

An indirect solid-phase enzyme-immunoassay (EIA) was developed for the detection of poliovirus antigen. Poliovirus antigen was obtained in LLCMK-2 cell culture and used to immunize rabbits and guinea pigs The poliovirus antibodies were evaluated by double immunodifusion in agar gel and neutralization test. Optimal concentrations of guinea pig and rabbit immunoglobulins were determined by checkerboard titration. Microtitre plates were coated with 15.0 ug/ml guinea pig anti-polio immunoglobulin and rabbit anti-polio immunoglobulin at the concentration of 7.94 ug/ml was used as detecting antibody. The performance of EIA was analysed from standard curve by measuring the coefficient of variation (CV) and the establishment of dose response relationship. The standard curve with eight different concentrations in eight replicates results in a CV between 2.1% to 7.8%. The dose response relationship was determined by simple linear regression (OD = 0,0124 x [Ag] + 0,4396) with a coefficient of correlation, R^2, equal to 96.40%. The assay detected a minimum of 2.3 ug/ml polio antigen.

INTRODUCTION

The approaches for laboratory diagnosis of virus infection have been the isolation of the agent in host systems and/or detection and measurement of antibody in host serum (Santos, 2002; Lennette, 1995; Engleberg, 1999). The development of virus infection laboratory diagnosis has been toward rapid methods capable to provide answer in less than 24 hours. The best methods should fulfill the five prerequisites of speed, simplicity, sensitivity, specificity and low cost. Tests for viral antigen, which provide a diagnosis from a single specimen, are rather important for many purposes. Solid-phase enzyme immunoassay (EIA) is one of the methods of choice.

Poliovirus, a member of family *Picornaviridae*, genus *Enterovirus*, was responsible for the tragic legacy of paralysis and deformity in the 1950s. The identification of polio relies mainly on the serum neutralization test (Nt) (Wahby, 2000; Payment et al., 1982). Because Nt test depends on living host system, it is costly, time consuming and the interpretation of neutralizing titers is influenced by subjective visual scoring of cytophatic effects (CPE). More rapid identification has been attempted using negative staining electron-microscopy (EM), immunofluorescence (IF) or immunoelectrophoresis (IEP). These techniques are not always applicable to screening of large number of samples (Payment et al., 1982).

The EM has the advantage of very rapid diagnosis but it is counteracted by the high cost of equipment, low detection limit (10^6 particles ml^{-1}), limited number of samples which can be processed and subjective assessment by the investigator (Czerny and Eichhorn, 1989). Assays based on IF are rapid and sensitive reactions, but a high background due to the autofluorescence of proteins and the difficulty to obtain objective results limit its possibilities.

Virus isolation can be hampered by several factors, such as, time consuming, cost and technical experience (Collins et al., 1988). The chance for virus isolation decreases with the duration of illness and the specimens must be preserved during transport to laboratory because of the loss of virus infectivity, cause of false negative results (Kok et al., 1998; Collins et al., 1988). The observation for specific CPE in inoculated cultures is labour intensive, particularly, for large number of samples and long time required for low virus titers to develop CPE (Kok et al., 1998; Payment et al., 1982).

The detection of viral antigen in clinical samples, as mentioned before, can be a rapid and economical alternative to cell culture.

EIA offers several advantages including speed, ease of performance and objective results (Deshpande, 1996a; Middeldorp et al., 1987; Gleaves et al., 1990). It is the most widely used of all immunological assay for antibodies and antigens, since large number of test can be performed in relative short time with maximum of sensitive and minimum technical expertise and equipment (van Weemen, 1985; Pillot, 1996).

This paper describes an EIA for the detection of poliovirus 1 antigens.

2.MATERIALS AND METHODS

VIRUS AND CELL CULTURE

Poliovirus type 1 obtained from American Type and Culture Collection (ATCC, 192-VR) was propagated in LLCMK-2 cell cultures. The cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Chem. Co, USA.) supplemented with 7% fetal bovine serum (Gibco BRL, USA), 100 ug/ml streptomycin

(Sigma Chem. Co, USA), 100 UI/ml penicillin (Sigma Chem. Co., USA) and 2.5 ug/ml fungizon (Bristol-Myers Squibb, Brazil).

ANTIGEN PRODUCTION AND PURIFICATION

The supernatant fluid of virus-infected cell cultures was harvested when cultures showed maximal CPE, usually 24-48 hours post-inoculation. The virus titer was determined by $TCID_{50}$ as 0.5 x 10^6/ml. The supernatant fluid of infected cell cultures was clarified by centrifugation at 10,000 x g for 30 min. Partial concentration of virus was carried out by the addition of polyethylene glycol (PEG) 6000 (8%, W/V) at 4°C overnight under agitation and followed by centrifugation at 10,000 x g for 30 min at 4°C. The resulting precipitate was resuspended in 10 ml PBS, pH 7.4, and 1.0-2.0 ml layered on a cushion of 20% sucrose in PBS, and ultracentrifuged in a Beckman SW41 rotor at 100,000 x g for 3 hours. The pellet was resuspended in 4 ml PBS. The protein content was determined by the method of Folin-Ciocalteau, and the material was stored at -20°C.

IMMUNIZATION OF ANIMALS

Rabbits and guinea pigs were inoculated five times at intervals of one week with 0.2 ml purified viral antigen suspension emulsified with an equal volume of Freund’s Complete Adjuvant. The animals were bled by cardiac puncture seven days after the final booster.

ABSORPTION OF SERA

Viral antisera free from LLCMK-2 cell antibodies were prepared by absorbing them with 10^5 cells/ml suspension (vol/vol), maintained overnight under constant agitation at 12°C followed by centrifugation at 1,000 x g for 10 min (Tyrrell, 1973).

DETERMINATION OF ANTIBODY TITERS

Double immunodiffusion in agar gel was used for screening pooled antibodies raised in rabbit and guinea pig against viral antigens, and was performed as previously described (Johnson, 1986).

Neutralization test was carried out as follows. Test sera were prepared in twofold dilution in cell culture maintenance medium. Thereafter, 100 ul volume of each dilution was mixed with an equal volume of $100TCID_{50}$ of virus and incubated at 37°C for 1 hour. The mixture was inoculated onto cell monolayers grown in 96-well tissue culture plates and incubated at 37°C for 5 days in 5% CO_2 atmosphere. The antibody endpoint (titer) was determined as the highest dilution of the serum that inhibits CPE.

PRECIPITATION OF IMMUNOGLOBULINS

A volume of hyperimmune serum pool was diluted with same volume of distilled water. Total immunoglobulin was precipitated by slowly addition of equal volume of ammonium sulfate 50% saturated followed by centrifugation at 1,000 x g for 10 min. The precipitate was dissolved in PBS and the precipitation process repeated twice. The final precipitate was dissolved in approximately 4 ml PBS and dialyzed overnight against PBS at 4°C. The protein content was determined by the method of Folin-Ciocalteau, and the material was stored at -20°C.

INDIRECT ENZYME IMMUNOASSAY PROCEDURE FOR CHECKERBOARD TITRATION

The procedure for the detection of viral antigens was established using an antigen capture EIA similar to that described by Ziegler et al. (1983). Optimal concentrations of guinea pig and rabbit immunoglobulins were determined by checkerboard titration on microtiter plates. Typically, protein concentration of 1 ug/ml is used to saturate the surface (Deshpande, 1996b); and should not exceed 10 ug/ml IgG for solid-phase adsorption (Porstmann and Kiessig, 1992). Thus, the guinea pig antibody concentrations were estimated as the established limit above. Briefly, flat bottom polystyrene microtiter plates (Hemobag, Brazil) were coated with 100 ul per well of guinea pig antibodies diluted at 1.5 ug/ml (half plate) and 15.0 ug/ml (half plate) in 0.1 M carbonate-bicarbonate buffer, pH 9.6, overnight, at 4°C, in a humidified chamber. Plates were washed twice with PBS, pH 7.2, containing 0.05% TWEEN 20 (PBS-T). The coated plates were blocked with 200 ul/well of blocking solution (PBS, pH 7.2, containing 2% skimmed milk powder and 0.05% TWEEN 20) for 90 min at 37°C. Plates were washed thrice with PBS-T throughout, unless otherwise indicated, and 100 ul/well in one column with PBS (blank), two columns for each viral antigen concentration at 0.03, 3.0 and 30.0 ug/ml as positive control were incubated at 37°C for 60 min. Plates were washed with PBS-T. Aliquots of 100 ul of rabbit antibody, diluted in a serial dilution from 127.0 ug/ml to 2.0 ug/ml, in blocking solution, pH 7.2, were added and the plates incubated at 37°C for 60 min. Incubation was followed by washing and the addition of 100 ul of goat anti-rabbit IgG peroxidase-conjugate (Sigma Chem. Co., USA) at 1:40,000 in blocking solution, pH 7.2, for 60 min at 37°C. Plates were washed and followed by the addition of 100 ul of substrate (0.03% H_2O_2) and chromogen, 400 ug/ml *o*-phenylenediamine dihydrochloride (Sigma Chem. Co.,

USA) diluted in 0.05 M citrate-phosphate buffer, pH 5.0. After 15 min at 25°C in light tight box, the reaction was stopped by the addition of 50 ul 2 N H_2SO_4 and the optic density (OD) read at 490 nm.

ANTIGEN STANDARD CURVE FOR DOSE RESPONSE

The standard curve was generated from measures of decreasing amounts of antigen concentration (300.0 ug/ml to 2.3 ug/ml) in eight replicates by previous protocol with the immunoglobulin concentrations optimized by checkerboard titration (15.0 ug/ml for capture antibody and 7.94 ug/ml for detector antibody).

STATISTICAL ANALYSIS OF DATA

The standard curve was used to estimate the dose-response relationship and the variation associated with the measurement of the analyte concentration.

The coefficient of variation, CV = (SD/mean) x 100, of each point of the standard curve was estimated by assaying this point eight times.

The dose response relationship was determined by linear regression to determine whether two or more variables are linearly related (Microsoft EXCEL).

The selection of data points for a given dilution curve was empirical. The analysis was based on the number of data points and the correlation coefficient (R^2). Normally, at least four data points are included and a R^2 equal or larger than 95%.

CLINICAL SPECIMENS

The five faecal specimens of children between one to two years of age were collected in June and July 2003 after the Nationwide Vaccination Day against Poliomyelitis campaign, maintained in –20°C, were supplied by the Laboratório

LAPAC (Ponta Grossa- PR). Four replicates of each sample diluted at 1:10 were tested by the previous optimized condition.

CUT-OFF

The cut-off value is defined as mean negative + 2.8 x standard deviation of negative specimens with a confidential interval of 99.8%, according to Porstmann and Kiessig (1992).

3.RESULTS

The presence of anti-polio immunoglobulins in guinea pig and rabbit sera was determined by double immunodiffusion in agar gel and neutralization test. The pattern of precipitin lines in double immunodiffusion test indicated a strong reaction against the purified polio antigens, on day 28 after the first injection, for both rabbit and guinea pig hyperimmune sera. A moderate response against cellular antigens was also observed. These hyperimmune sera evaluated by neutralization test demonstrated a titer of 1: 5,120 of neutralizing antibodies for both animals.

The determination of protein concentration of cellular and viral antigens and immunoglobulins was carried out to proceed checkerboard titration and standard curve analysis.

The concentrations of protein in LLCMK-2 and purified polio antigen preparations were estimated in 7.3 mg/ml and 3.3 mg/ml, respectively, and for antisera in 12.7 mg/ml and 15.0 mg/ml, respectively, for guinea pig and rabbit.

Optimal concentrations of capture and detector immunoglobulins were determined by checkerboard titration against the antigen used for immunization (Schoenthaler and Kapil, 1999; Deshpande, 1996b; Voller and Bidwell, 1986). Each combination of capture and detector antibodies was tested in the presence of negative, 0.03 ug/ml, 3.0 ug/ml and 30.0 ug/ml antigen samples. The optimum immunoglobulins concentrations were those giving the greastest and most reproducible changes in signal with increasing antigen concentration. The initial analysis indicated high background due to an excessive non-specific binding (NSB) hampering distinction of a positive signal derived from the analyte against the blank values. Increasing blocking agent concentration from 1% to 2%, we minimized this type of NSB. Increasing the number of washes also alleviated this problem to a large degree. The concentration of guinea pig antibody (capture) of 15.0 ug/ml demonstrated a better result than 1.5 ug/ml. The slope of the curve indicated that suitable results would be achieved by the use of 15.0 ug/ml of capture antibody and 7.94 ug/ml of rabbit antibody (detector).

By the standard curve, we could evaluate the precision and the quantitative aspect of the assay. The precision, also referred as reproducibility, is a statistical measure of variation between repeated determination of the same sample. The statistical index used to express the precision profile is the coefficient of variation, calculated as following, CV = (Standard Deviation/Mean) x 100 at a particular antigen level. Different concentrations of antigen were used to evaluate the performance of the method on the same day in eight replicates and the CV varied from 2.1 to 7.8 %.

When evaluating the dose response by standard curve for decreasing concentration of reference antigens, a sigmoid curve was obtained. Simple linear regression was used to determine if the assay showed a statistically significant dose response

relationship in which the OD was directly proportional to the antigen concentrations (Wilbur, 1993). Antigen concentrations chosen empirically was analyzed to reach the best correlation (R^2) between OD and antigen concentration. The linear interval occurred between 2.3 ug/ml and 37.5 ug/ml and resulted in a dose response relationship represented by OD = 0.0124 x [Ag] + 0.4396, with the coefficient of correlation, R^2, equal to 96.4%.

In the optimized condition, our assay detected cell antigen (LLCMK-2) only at levels higher than 730 ug/ml indicating that the assay had a low level of cross-reaction with cell antigen.

Our assay detected a minimum of 2.3 ug/ml polio antigen.

The clinical samples resulted in OD of 0.146, 0.164, 0.201, 0.227, and 0.356, while the cut-off value resulted in an OD of 0.141.

4.DISCUSSION

We developed an adaptation of an EIA for the detection of polio antigens

Specific antibodies for polio antigens were demonstrated by double immunodifusion in agar gel and titered by neutralization tests suggesting the efficacy of viral purification and immunization procedures.

Optimal concentrations of rabbit and guinea pig immunoglobulin were evaluated by checkerboard titration and the concentrations selected on the basis of maximal sensitivity and minimum non-specific reactions.

The solid phase has a limited capacity for binding proteins. Most microtiters plates are capable of binding approximately 0.4 pg/cm^2, and when antibody concentration

exceeds 1 ug/cm^2, unstable bi- and polylayers are formed, from which analyte molecules binding to second layers are removed as antigen-antibody complexes by bound-free separation (Deshpande, 1996b; Porstmann and Kiessig, 1992). Thus, we preferred to test first the minimum concentration of capture antibody that allowed high sensitivity without exceeding the immunosorbent capacity of solid phase. High concentrations of detector antibody have also a disadvantage of the increased background that offsets the benefits of the signal magnitude.

Precision is probably the most important technical aspect of an assay performance. The performance of the assay was demonstrated to be good by determining the CV. This was over the measurement range of EIA of < 15%, whereas 30% is the maximum acceptable value for repeatability of a diagnostic test (El Idrissi and Ward, 1992; Porstmann and Kiessig, 1992; Deshpande, 1996b; Gubbels et al., 2000). Our assay demonstrated a CV varying from 2.1 to 7.8 %.

Our assay detected a minimum of 2.3 ug/ml polio antigen. Most of EIAs detect antigen at level of 1 ng/ml (Deshpande, 1996b). According to our data, it is suggested that there is still a significant background or the avidity of antibody is not sufficient to detect lower levels of the antigens. Improving the final detection system with, such as, avidin-biotin or coupled enzyme cascade systems, we could solve it (Grandien, 1996; Jackman, 1986).

In conclusion, we described an antigen detection test for polio antigens, which has a potential use in diagnostic procedures, and also for monitoring the antiviral properties of substances in the replication of the virus.

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ANEXOS

ANEXO I: TABELAS E GRÁFICOS

MICROPLACAS DE CALIBRAÇÃO DOS REAGENTES DO EIE

Gráfico 1.1: Microplaca de calibração, coberta com 0.9 ug/ml de anticorpo de cobaia, em diferentes concentrações de anticorpos anti-BHV-1 de coelho (Ac coelho).

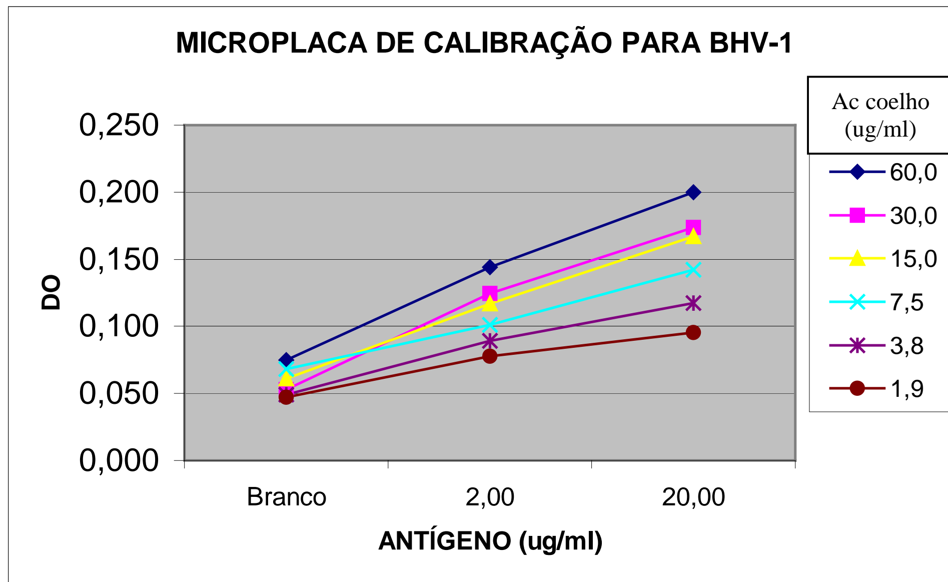
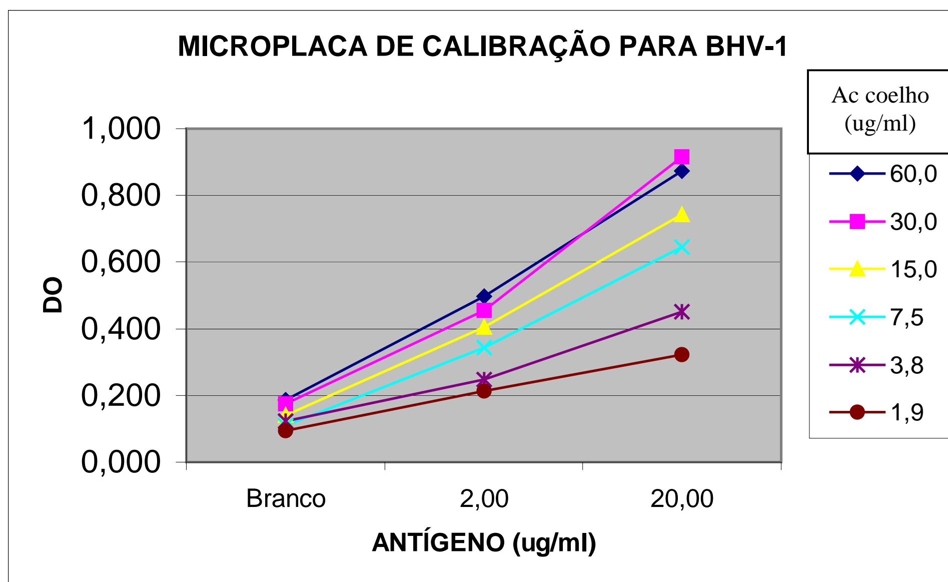


Gráfico 1.2: Microplaca de calibração, coberta com 9.0 ug/ml de anticorpo de cobaia, em diferentes concentrações de anticorpos anti-BHV-1 de coelho (Ac coelho).



MICROPLACAS DE CALIBRAÇÃO DOS REAGENTES DO EIE

Gráfico 1.3: Microplaca de calibração, coberta com 1.5 ug/ml de anticorpo de cobaia, em diferentes concentrações de anticorpos anti-pólio de coelho (Ac coelho).

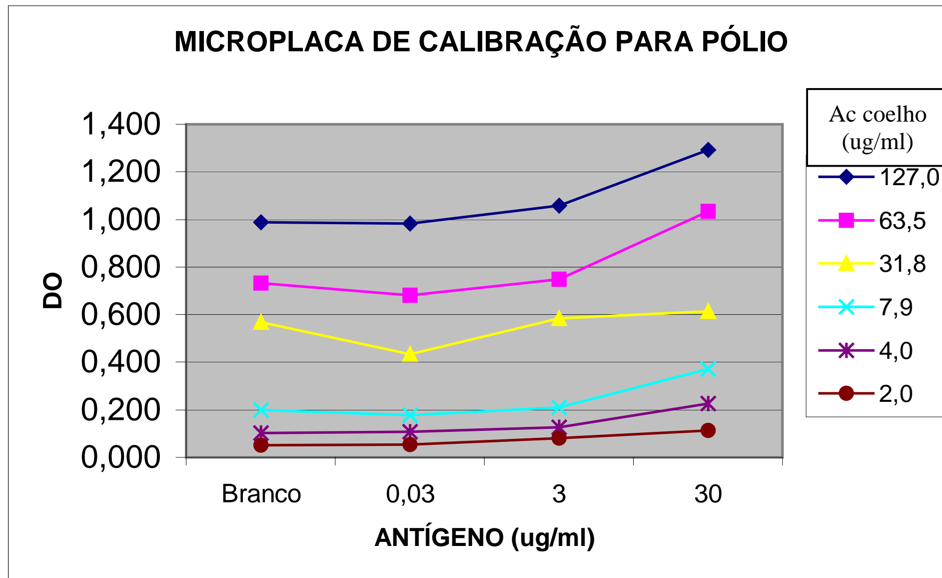
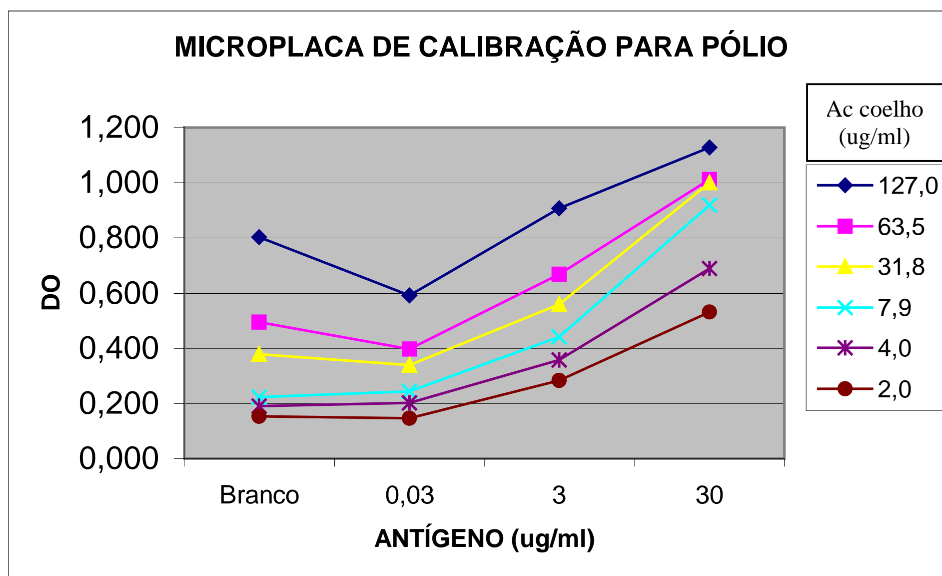


Gráfico 1.4: Microplaca de calibração, coberta com 15.0 ug/ml de anticorpo de cobaia, em diferentes concentrações de anticorpos anti-pólio de coelho (Ac coelho).



ANÁLISE DA PRECISÃO DO EIE

Gráfico.2.1: Coeficiente de variação do EIE padronizado em diferentes concentrações de antígeno BHV-1.

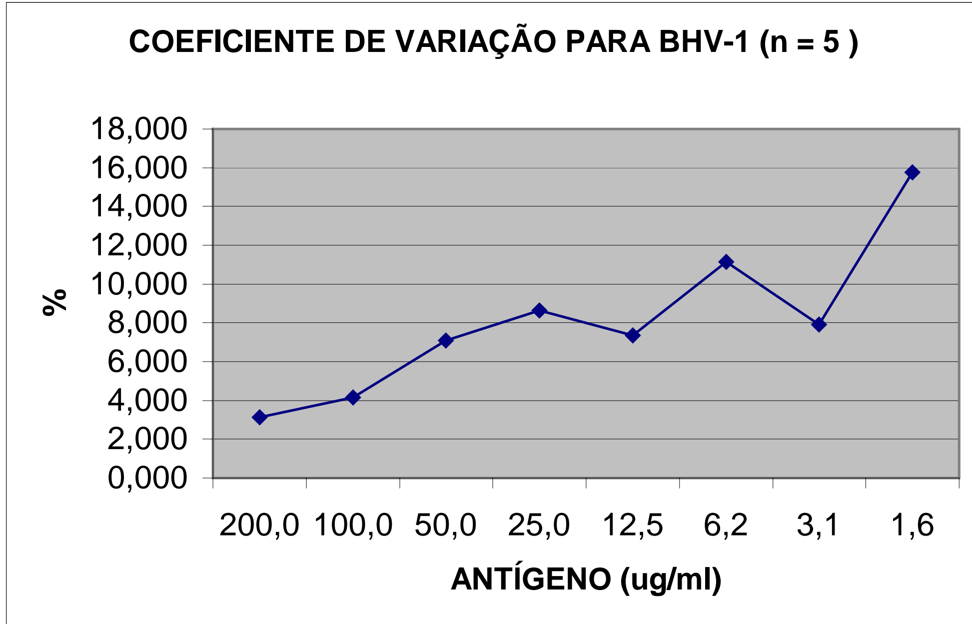
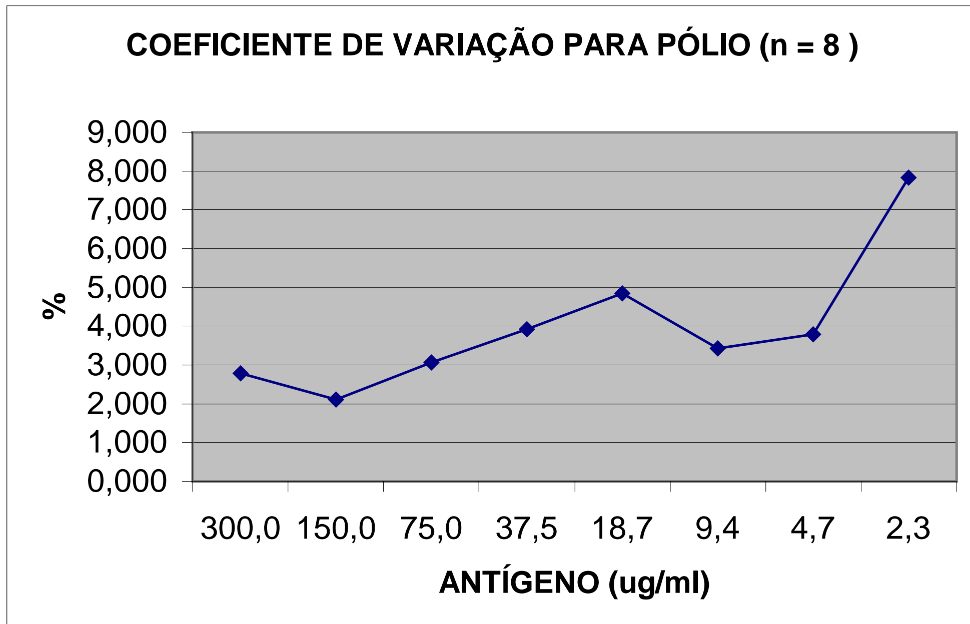


Gráfico 2.2: Coeficiente de variação do EIE padronizado em diferentes concentrações de antígeno pólio.



CURVA DE CALIBRAÇÃO DO EIE

Gráfico 3.1: Curva de calibração do EIE padronizado, utilizando diferentes concentrações de antígeno BHV-1.

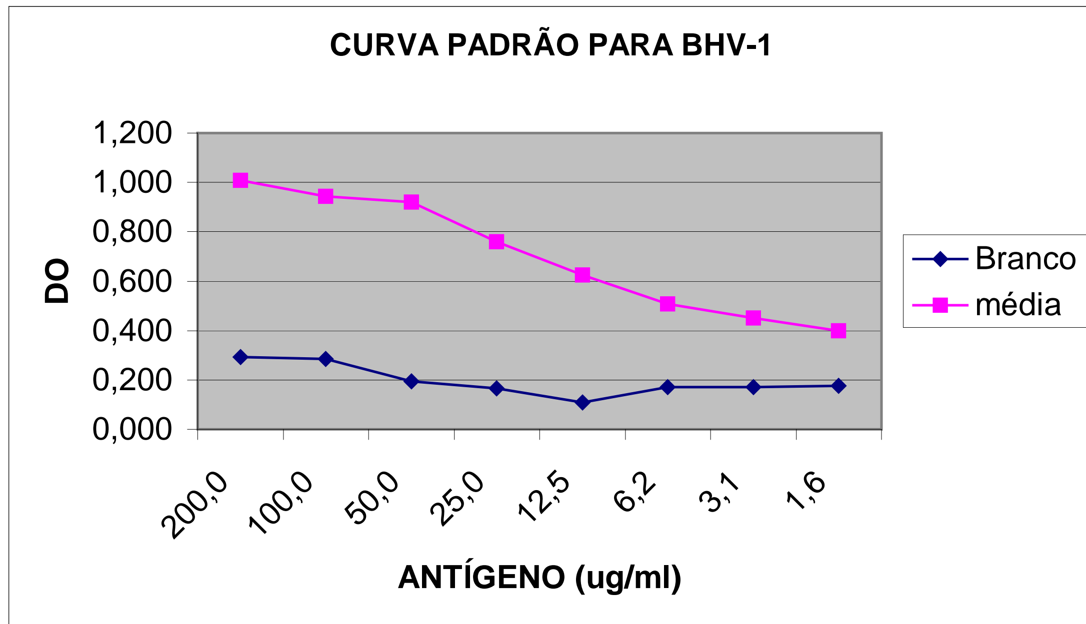
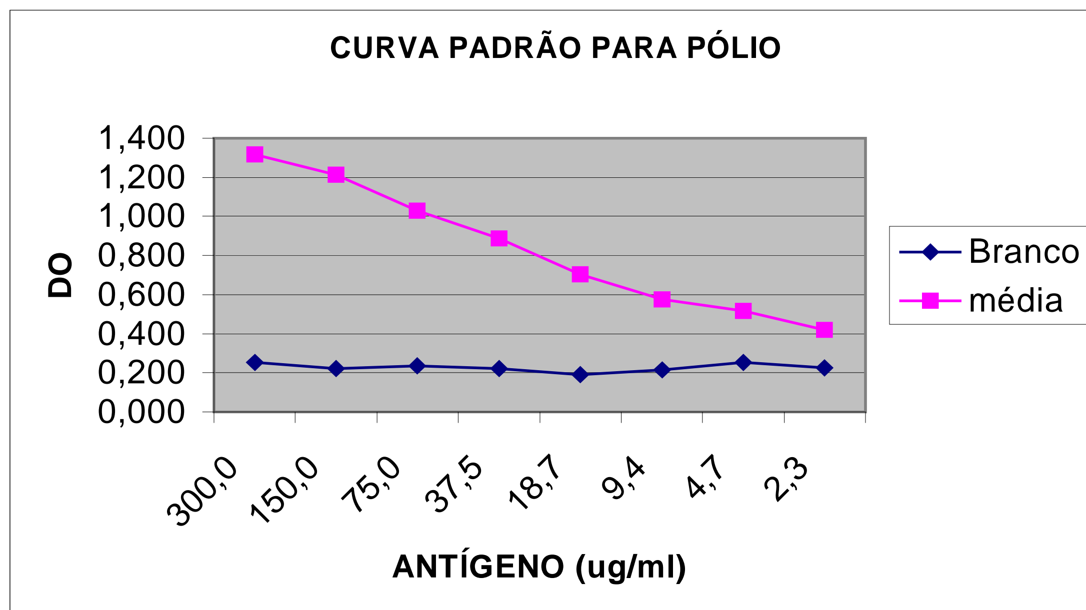


Gráfico 3.2: Curva de calibração do EIE padronizado, utilizando diferentes concentrações de antígeno pólio.



ANÁLISE QUANTITATIVA DO EIE

Gráfico 4.1: Curva padrão por regressão linear simples na determinação da relação dose-resposta para BHV-1.

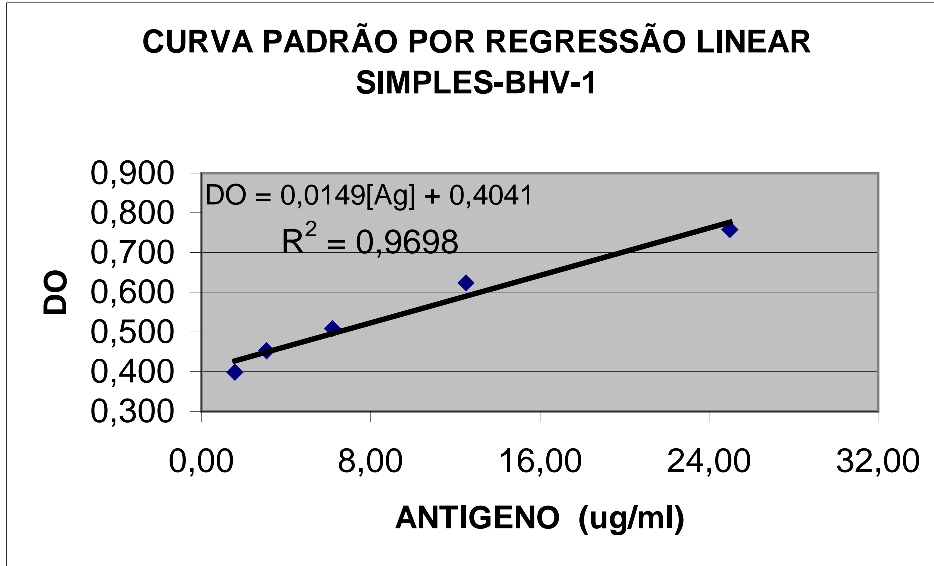
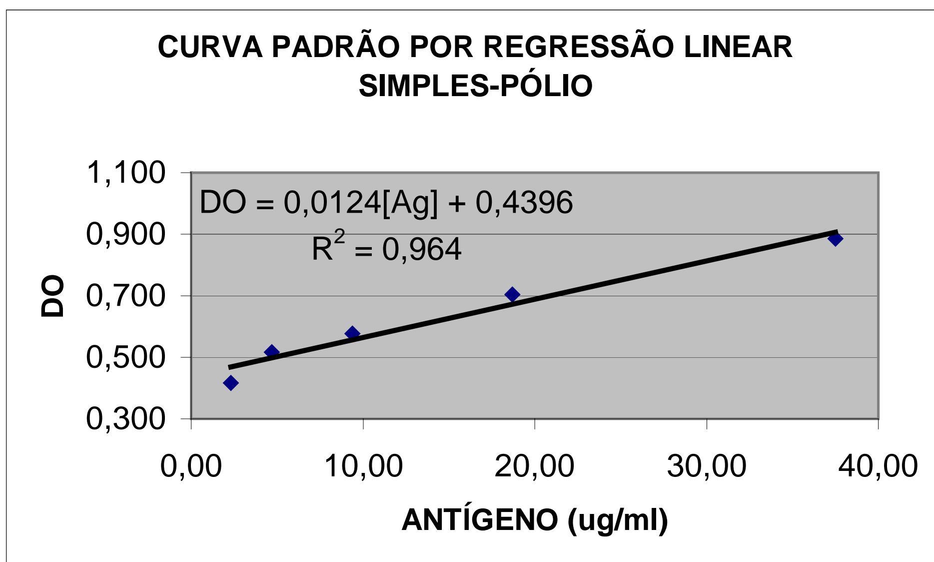


Gráfico 4.2: Curva padrão por regressão linear simples na determinação da relação dose-resposta para pólio.



ANÁLISE DA PERFORMACE DO EIE PARA AS AMOSTRAS DE CAMPO

Gráfico 5.1: Amostras bovinas positivas para BHV-1 pelo EIE padronizado.

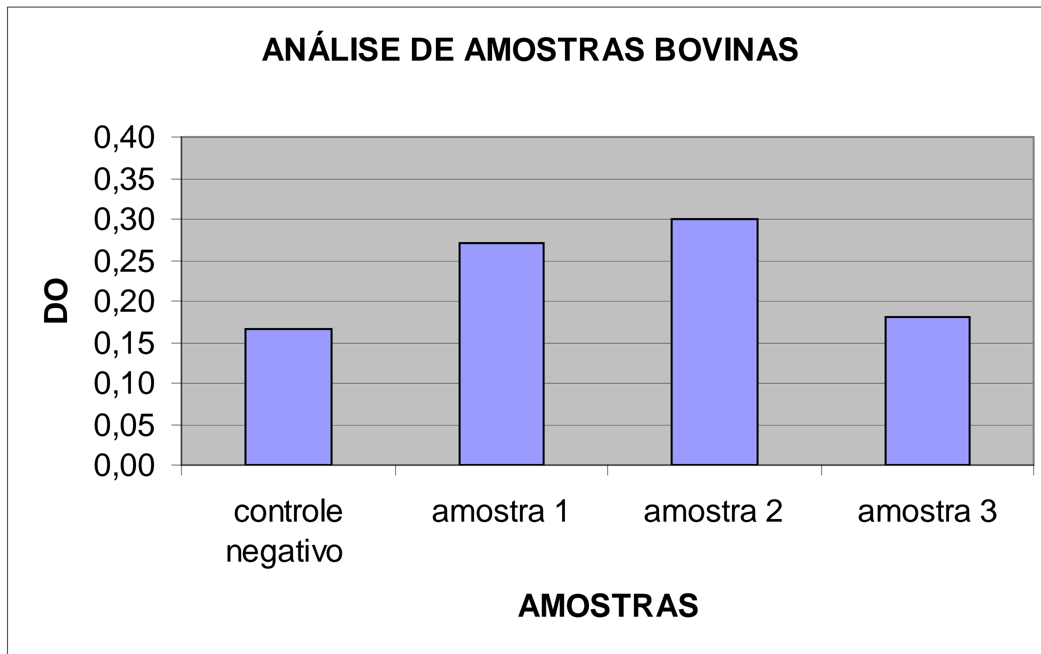
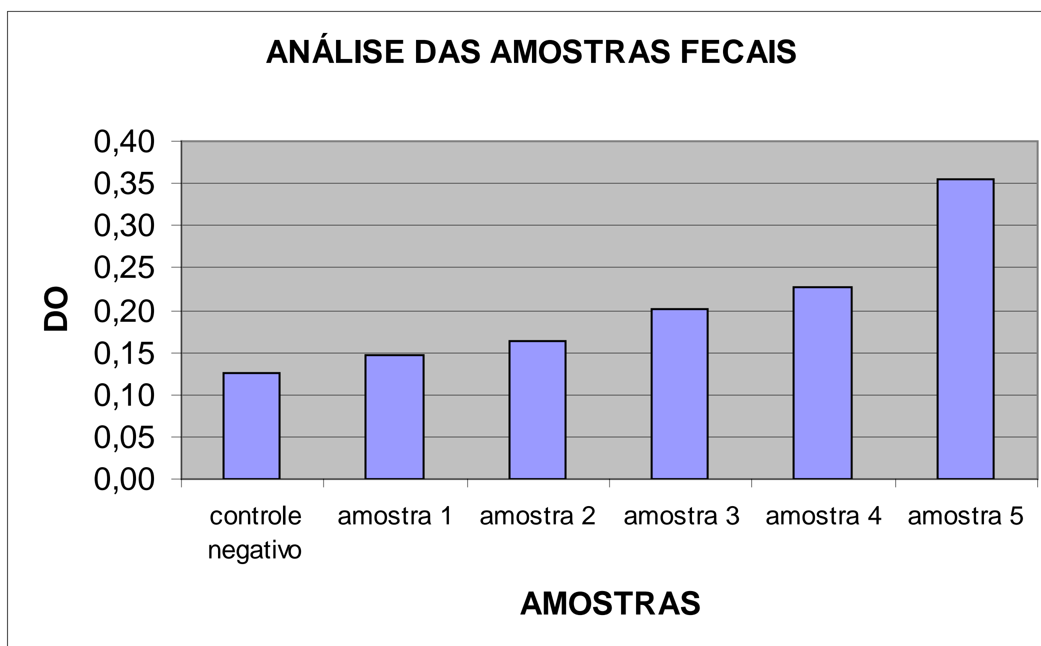


Gráfico 5.2: Amostras fecais positivas para pólio pelo EIE padronizado.



REDUÇÃO DA REAÇÃO DE FUNDO (BACKGROUND)

Gráfico 6.1: Influência da concentração do leite em pó desnatado (1 e 2%) na reação de fundo em microplacas, cobertas com 9.0 ug/ml de anticorpo de cobaia, com diferentes concentrações de anticorpo de coelho.

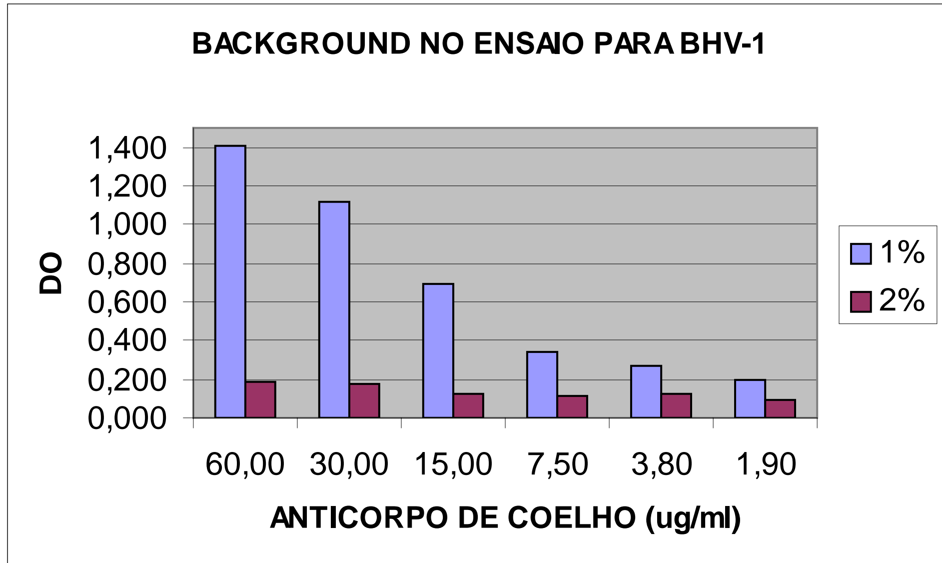


Gráfico 6.2: Influência da concentração do leite em pó desnatado (1 e 2%) na reação de fundo em microplacas, cobertas com 15.0 ug/ml de anticorpo de cobaia, com diferentes concentrações de anticorpo de coelho.

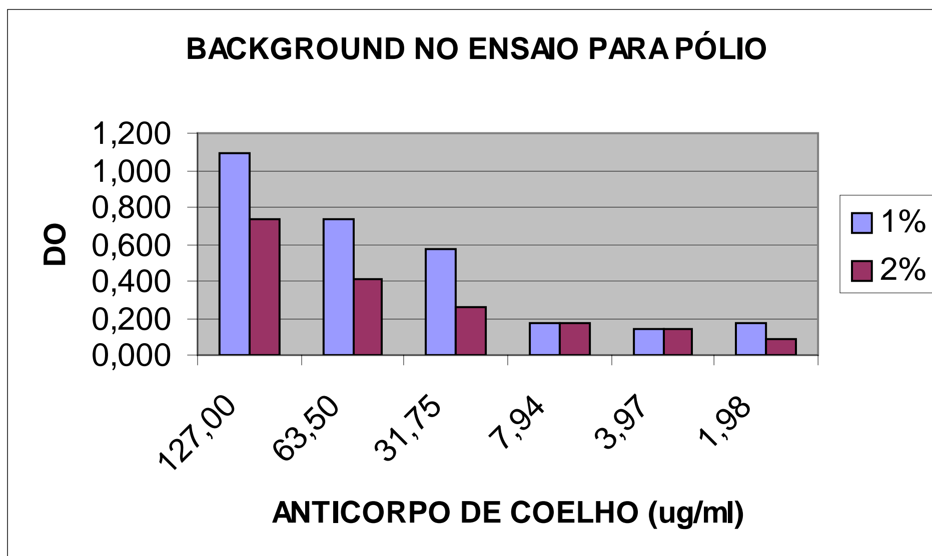


Tabela 1.1: CURVA DE CALIBRAÇÃO PARA PÓLIO COM EIE PADRONIZADO

ANTíGENO (ug/ml)	BRANCO	MÉDIA	SD	CV (%)
300.0	0.249	1.316	0.037	2.791
150.0	0.232	1.214	0.026	2.105
75.0	0.220	1.030	0.032	3.061
37.5	0.219	0.886	0.035	3.930
18.7	0.202	0.704	0.034	4.853
9.4	0.220	0.576	0.020	3.423
4.7	0.248	0.517	0.020	3.797
2.3	0.246	0.418	0.033	7.837

Tabela 1.2: CURVA DE CALIBRAÇÃO PARA BHV-1 COM EIE PADRONIZADO

ANTíGENO (ug/ml)	BRANCO	MÉDIA	SD	CV (%)
200.0	0.293	1.009	0.032	3.144
100.0	0.286	0.942	0.039	4.144
50.0	0.195	0.919	0.065	7.072
25.0	0.165	0.758	0.066	8.643
12.5	0.110	0.624	0.046	7.366
6.2	0.171	0.509	0.057	11.128
3.1	0.170	0.452	0.036	7.908
1.6	0.176	0.398	0.063	15.768

AMOSTRAS

Tabela 2.1: poliovírus

	NEGATIVO	AMOSTRA 1	AMOSTRA 2	AMOSTRA 3	AMOSTRA 4	AMOSTRA 5
	0.127	0.147	0.152	0.190	0.234	0.392
	0.122	0.144	0.170	0.194	0.216	0.334
	0.133	0.144	0.205	0.177	0.243	0.356
	0.122	0.149	0.128	0.243	0.215	0.342
MÉDIA	0.126	0.146	0.164	0.201	0.227	0.356

Tabela 2.2: BHV-1

	NEGATIVO	AMOSTRA 1	AMOSTRA 2	AMOSTRA 3
	0.183	0.255	0.280	0.161
	0.164	0.248	0.310	0.174
	0.169	0.270	0.297	0.166
	0.178	0.293	0.304	0.197
	0.174	0.284	0.324	0.186
	0.143	0.256	0.287	0.193
	0.156	0.283	0.280	0.177
	0.163	0.282	0.318	0.181
MÉDIA	0.166	0.271	0.300	0.179

ANEXO II - SOLUÇÕES

SOLUÇÃO TAMPÃO FOSFATO-SALINA

NaCl..8,0g
KCl...0,2g
Na_2HPO_4..1.15g
KH_2PO_4...0,2g
H2O destilada...1000ml

TAMPÃO CARBONATO-BICARBONATO 0,1M pH 9,6

Na_2CO_3...1,59g
$NaHCO_3$..2,93g
H_2O destilada...qsp 1000ml

SOLUÇÃO DE BLOQUEIO

Leite desnatado...2g
TWEEN
20..0,05ml
PBS...qsp 100ml
Ajustar o pH para 7.2 com HCl 1N ou NaCl 1N.e estocar a 4°C

TAMPÃO DE LAVAGEM (PBS-TWEEN 20)

TWEEN 20..1ml
PBS...2000ml
Ajustar o pH para 7.2 com HCl 1N ou NaCl 1N.e estocar a 4°C

TAMPÃO CITRATO-FOSFATO

Na_2HPO_4..1,37g
Ácido cítrico 9,33g
H_2O..qsp 200ml
Ajustar o pH para 5.0 com HCl 1N ou NaCl 1N.e estocar a 4°C

SOLUÇÃO DE ÁCIDO SULFÚRICO 2N

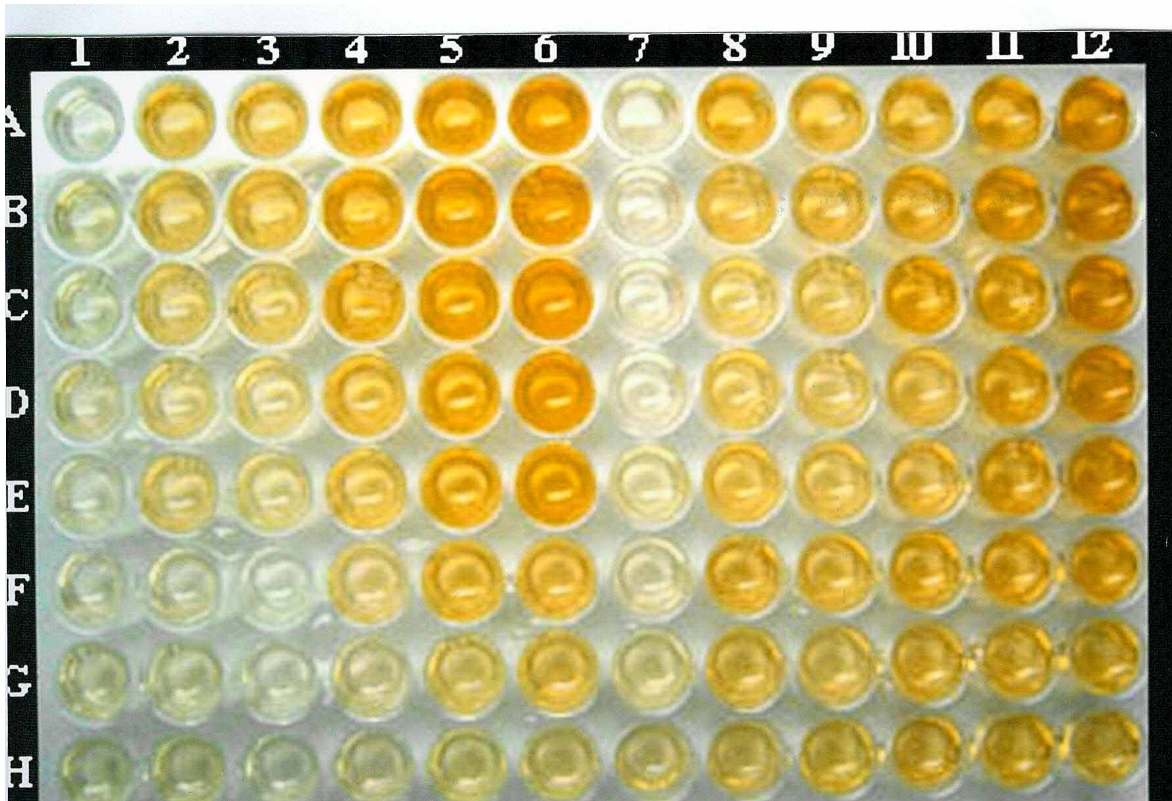
H_2SO_4...5,37ml
H_2O..qsp 100ml

SOLUÇÃO DE DIIDROCLORIDRATO DE ORTO-FENILAMINA DIAMINA (OPD)

OPD...20mg
H_2O_2 30 % (vol)..20ul
Tampão citrato-fosfato..50ml

Dissolver o OPD em tampão fosfato-citratro, adicionar H_2O_2 e colocar imediatamente nos orifícios da placa. Preparar apenas no momento do uso. Deixar reagir por 15 min. no escuro à temperatura de 28 a 30 ^{o}C. Adicionar 50 ul de H_2SO_4 aos orifícios da placa para paralisar a reação. A leitura da absorbância é realizada no comprimento de onda de 490 nm.

ANEXO III: ESQUEMA DE MICROPLACA PARA A CALIBRAÇÃO DAS CONCENTRAÇÕES ÓTIMAS DE IMUNOGLOBULINAS DE CAPTURA E DETECÇÃO



Linhas: anticorpo de detecção (coelho)
A = 130.0 ug/ml
B = 65.0 ug/ml
C= 32.5 ug/ml
D = 16.25 ug/ml
E= 8.12 ug/ml
F = 4.06 ug/ml
G = 2.03 ug/ml
H = 1.01 ug/ml

Colunas: antígeno
1 e 7 = Branco
2 e 8 = 2.34 ug/ml
3 e 9 = 4.69 ug/ml
4 e 10 = 18.75 ug/ml
5 e 11 = 75.0 ug/ml
6 e 12 = 300.0 ug/ml

Divisão da placa: Anticorpo de captura (cobaia)
1-6 = 1.5 ug/ml
7-12 = 15.0 ug/ml