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(57) Resumo: COMPOSIÇÃO VACINAL CONTRA LEISHMANIOSE VISCERAL CANINA, PEPTíDEOS SINTÉTICOS E USO. A presente tecnologia descreve uma composição vacinal para prevenção e ou tratamento da leishmaniose visceral canina, utilizando como antígenos, peptideos expressos na superfície externa de bacteriófagos não nocivos ao hospedeiro mamífero, ou sintetizados, sendo utilizados isolados ou associados. A tecnologia descreve também o uso desses peptídeos na preparação de das composições vacinais contra leishmaniose visceral. Esses peptideos foram selecionados pela técnica phage display e induzem resposta imune específica do tipo Th1, destacada pela maior produção de 1FN-gama

COMPOSIÇÃO VACINAL CONTRA LEISHMANIOSE VISCERAL CANINA, PEPTÍDEOS SINTÉTICOS E USO

A presente tecnologia descreve uma composição vacinal para prevenção e/ou tratamento da leishmaniose visceral canina, utilizando como antígenos, peptídeos expressos na superfície externa de bacteriófagos não-nocivos ao hospedeiro mamífero, ou sintetizados, sendo utilizados isolados ou associados. A tecnologia descreve também o uso desses peptídeos na preparação de vacinas contra leishmaniose visceral. Esses peptídeos foram selecionados pela técnica *phage display* e induzem resposta imune específica do tipo Th1, destacada pela maior produção de IFN-gama.

As leishmanioses são doenças endêmicas em diversos países no mundo. Estima-se que cerca de 380 milhões de pessoas estejam expostas aos riscos de infecção e que 12 milhões sejam clinicamente afetadas pela doença. Segundo levantamentos da Organização Mundial de Saúde (OMS), cerca de 2.0 milhões de novos casos da doença ocorrem a cada ano, dos quais cerca de 1.0 a 1.5 milhão correspondem a casos de leishmaniose tegumentar (LT) e cerca de 500.000 casos de leishmaniose visceral (LV) são registrados [World Health Organization. Control of the leishmaniasis: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22-26 March 2010. WHO Technical Report Series. (949). http://whqlibdoc.who.int/trs/WHO_TRS_949_eng.pdf. 2010].

A gravidade da doença no hospedeiro mamífero, no qual se enquadram o homem e o cão, alcança desde uma lesão cutânea única, no local da picada, até a forma visceral da doença, que pode ser fatal quando na forma aguda e se não tratada. O cão é considerado o principal reservatório doméstico do parasita e é tido como uma importante fonte de transmissão entre o vetor flebotomíneo e o hospedeiro mamífero [Grimaldi, G. & Tesh, R.B. Leishmaniasis of the New World: current concepts and implications for future research. *Clin. Microbiol. Rev.* 6;230–50, 1993].

O diagnóstico da LV é dificultado pela semelhança dos sintomas clínicos com outras doenças, também comuns em nosso meio. Em paralelo, testes

laboratoriais baseiam-se na detecção de antígenos e/ou anticorpos específicos aos parasitas em amostras de soro dos pacientes. Nesses casos, a Análise de Imunoadsorção por Ligação Enzimática (ELISA), a Reação de Imunofluorescência Indireta (IFAT) e o Teste de Aglutinação Direta (DAT) são utilizados, porém, dentre alguns problemas relatados, não apresentam a capacidade de diferenciar pacientes com a doença subclínica, com a doença na forma ativa ou com a mesma já curada [Tavares CA, Fernandes AP, Melo MN. Molecular diagnosis of leishmaniasis. *Expert Rev Mol Diagn.* 3;657-67, 2003].

No caso da LV canina (LVC), o diagnóstico apresenta-se dificultado por fatores como a especificidade e/ou sensibilidade variáveis dos antígenos utilizados. Nesse caso, a ocorrência de um elevado número de resultados falso-positivos em animais saudáveis, porém, residentes em áreas endêmicas da doença, bem como em animais portando outras doenças, tais como doença de Chagas, babesiose, toxoplasmose, rickettsiose e erlichiose; e a ocorrência de resultados falso-negativos, como em animais infectados, porém, com baixos títulos de anticorpos anti-*Leishmania*; têm criado grandes dificuldades para a interpretação correta dos testes sorológicos nos animais [Scalone A, Luna R, Oliva G, Baldi L, Satta G, Vesco G, Mignone W, Turilli C, Mondesire RR, Simpson D, Donoghue AR, Frank GR, Gradoni L.. Evaluation of the *Leishmania* recombinant K39 antigen as a diagnostic marker for canine leishmaniasis and validation of a standardized enzyme-linked immunosorbent assay. *Vet. Parasitol.* 104;275–85, 2002. Mettler M, Grimm F, Capelli G, Camp H, Deplazes D. Evaluation of enzyme-linked immunosorbent assays, an immunofluorescent-P.antibody test, and two rapid tests (immunochromatographic-dipstick and gel tests) for serological diagnosis of symptomatic and asymptomatic *Leishmania* infections in dogs. *J. Clin. Microbiol.* 43;5515–19, 2005. Figueiredo FB, Madeira MF, Nascimento LD, Abrantes TR, Mouta-confort E, Passos SRL, Schubach TMP. Canine visceral leishmaniasis: study of methods for the detection of IgG in serum and eluate samples. *Rev Inst. Med. Trop. São Paulo* 52;193–96, 2010]. O diagnóstico definitivo é realizado por testes parasitológicos, com a detecção dos parasitas, nos quais se realizam culturas *in vitro* de aspirados do

baço, fígado e medula óssea; locais que abrigam os parasitas quando o hospedeiro mamífero está infectado [Sundar. S & Rai, M. Laboratory diagnosis of visceral Leishmaniasis. *Clin Diagn Lab Immunol.* 9;951-958, 2002. Tavares CA, Fernandes AP, Melo MN. Molecular diagnosis of leishmaniasis. *Expert Rev Mol Diagn.* 3;657-67, 2003].

As medidas de controle empregadas visam, principalmente, à interrupção do ciclo biológico do parasita. Entretanto, o número de espécies de *Leishmania*, o caráter zoonótico da doença e a manutenção do parasita no ciclo silvestre dificultam a adoção de medidas efetivas de controle [Tesh RB. Control of zoonotic visceral leishmaniasis: is it time to change strategies? *Am J Trop Med Hyg.*52;287-92, 1995]. O controle do vetor pode ser realizado pela aplicação de inseticidas no ambiente doméstico e peridoméstico; entretanto, sua utilização apresenta relativa eficácia [Tesh RB. Control of zoonotic visceral leishmaniasis: is it time to change strategies? *Am J Trop Med Hyg.*52;287-92, 1995]. No que se refere à LVC, a eliminação de cães infectados e/ou animais soropositivos para antígenos de *Leishmania*, constitui-se como a medida de controle mais adotada pelos órgãos de Saúde competentes [Tesh RB. Control of zoonotic visceral leishmaniasis: is it time to change strategies? *Am J Trop Med Hyg.*52;287-92, 1995]. A eliminação de reservatórios silvestres, como marsupiais e roedores, não é considerada uma medida executável e ecologicamente correta, pois existe a possibilidade de adaptação do parasita a outros reservatórios existentes no ambiente [Grimaldi, G. & Tesh, R.B. Leishmaniasis of the New World: current concepts and implications for future research. *Clin. Microbiol. Rev.* 6;230–50, 1993. Gramiccia M, Gradoni L. The current status of zoonotic leishmaniases and approaches to disease control. *Int J Parasitol.* 35(11-12):1169-80. 2005]. Assim a melhor forma de um controle preventivo da doença seria com o uso de vacinas.

Em modelos murinos, após a cura da leishmaniose causada pela espécie *Leishmania major*, os animais adquirem imunidade duradoura contra a re-infecção [Afonso LC & Scott P. Immune responses associated with susceptibility of C57BL/10 mice to *Leishmania amazonensis. Infect. & Immun.* 61;2952–59, 1993]. Tal fato tem estimulado o desenvolvimento de pesquisas

visando à obtenção de antígenos vacinais que possam ser utilizados como uma medida efetiva de controle da doença. Existem antígenos que vêm sendo avaliados nas últimas décadas e que preenchem, parcialmente, requisitos para serem empregados como candidatos à vacina [Stäger S, Smith DF, Kaye PM. Immunization with a recombinant stage-regulated surface protein from *Leishmania donovani* induces protection against visceral leishmaniasis. *J Immunol* 165;7064–71, 2000. Dondji B, Perez-Jimenez E, Goldsmith-Pestana K, Esteban M, McMahon-Pratt D. Heterologous prime – boost vaccination with the LACK antigen protects against murine visceral leishmaniasis. *Infect Immun* 73;5286–89, 2005. Chávez-Fumagalli MA, Costa MA, Oliveira DM, Ramírez L, Costa LE, Duarte MC, Martins VT, Oliveira JS, Bonay P, Alonso C, Tavares CAP, Soto M, Coelho E A F. Vaccination with the *Leishmania infantum* ribosomal proteins induces protection in BALB/c mice against *Leishmania chagasi* and *Leishmania amazonensis* challenge. *Microbes Infect* 12;967–77, 2010. Agallou M, Smirlis D, Soteriadou KP, Karagouni E. Vaccination with *Leishmania* histone H1-pulsed dendritic cells confers protection in murine visceral leishmaniasis. *Vaccine* 30;5086–93, 2012]. Porém, devido às dificuldades relacionadas à obtenção de um antígeno que seja eficaz contra mais de uma espécie, que seja de fácil e rápida produção e que não estimule a produção de anticorpos específicos ao parasita nos animais imunizados, como por outros motivos; ainda não há disponível no mercado, uma vacina completamente eficaz no combate ao parasita.

Em relação à resposta imune na LV, a resistência à infecção parece estar relacionada com uma resposta imune específica do tipo celular do tipo Th1, destacada pela produção de IFN-gama, por parte do hospedeiro mamífero. O mecanismo imunológico efetor responsável pelo controle dos parasitas parece ser decorrente da ativação de macrófagos, via produção de níveis elevados dessa citocina [Scott P. Development and regulation of cell-mediated immunity in experimental leishmaniasis. *Immunol Res.* 27;489-98, 2003].

A utilização de modelos murinos em estudo de vacinação nas leishmanioses permitiu a identificação de dois subtipos distintos de linfócitos T

que produzem e secretam citocinas capazes de induzir funções efetoras diferentes. Os estudos que utilizaram como base o modelo murino de infecção por *L. major* em camundongos BALB/c, proposto por Sacks & Noben-Trauth (2002) definiram o paradigma Th1/Th2 de resistência e susceptibilidade, respectivamente, à infecção e o papel de citocinas como o IFN-gama e a interleucina-4 (IL-4), respectivamente, no desenvolvimento de linhagens de células Th1 e Th2 [Sacks D & Noben-Trauth N. The immunology of susceptibility and resistance to Leishmania major in mice. *Nat Rev Immunol.* 2;845-58, 2002].

Na LV murina, o perfil de resposta Th1 pode ser reprimido pela produção de citocinas como IL-10. Neste sentido, citocinas tidas como de resposta Th2, como IL-4 e IL-10, parecem estar relacionadas à desativação de macrófagos e no estabelecimento da doença nos animais, enquanto que citocinas como IFN-gama e IL-12 estariam relacionadas com o fenótipo de proteção contra a mesma [Zanin FH, Coelho EAF, Tavares CA, Marques-da-Silva EA, Silva Costa MM, Rezende SA, Gazzinelli RT, Fernandes AP. Evaluation of immune responses and protection induced by A2 and nucleoside hydrolase (NH) DNA vaccines against *Leishmania chagasi* and *Leishmania amazonensis* experimental infections. *Microbes Infect* 9;1070–77, 2007. Chávez-Fumagalli MA, Costa MA, Oliveira DM, Ramírez L, Costa LE, Duarte MC, Martins VT, Oliveira JS, Bonay P, Alonso C, Tavares CAP, Soto M, Coelho E A F. Vaccination with the *Leishmania infantum* ribosomal proteins induces protection in BALB/c mice against *Leishmania chagasi* and *Leishmania amazonensis* challenge. *Microbes Infect* 12;967–77, 2010. Martins VT, Chávez-Fumagalli MA, Costa LE, Canavaci AM, Lage PS, Lage DP, Duarte MC, Valadares DG, Magalhães RD, Ribeiro TG, Nagem RA, Da Rocha WD, Régis WC, Soto M, Coelho EA, Fernandes AP, Tavares CA. Antigenicity and protective efficacy of a *Leishmania* amastigote-specific protein, member of the super-oxygenase family, against visceral leishmaniasis. *PLoS Negl Trop Dis.* 7;e2148, 2013].

Em estudos de avaliação de candidatos vacinais na LV murina, com vistas à aplicação futura como vacinas na LV canina e humana, os imunógenos vacinais são administrados em camundongos, associados a adjuvantes de

resposta imune do tipo Th1, os animais, após certo tempo, são então infectados com *Leishmania* e, após algumas semanas, células do baço (esplenócitos) desses animais são retiradas, cultivadas e estimuladas *in vitro* com os antígenos utilizados nas imunizações para a avaliação da imunogenicidade dos mesmos. Nesse caso, citocinas como IFN-gama e IL-12, marcadores de resposta Th1, e IL-4 e IL-10, marcadores de resposta Th2; têm seus níveis determinados, sendo então realizada uma comparação entre tais níveis obtidos com as cargas parasitárias detectadas nos animais [Martins VT, Chávez-Fumagalli MA, Costa LE, Canavaci AM, Lage PS, Lage DP, Duarte MC, Valadares DG, Magalhães RD, Ribeiro TG, Nagem RA, DaRocha WD, Régis WC, Soto M, Coelho EA, Fernandes AP, Tavares CA. Antigenicity and protective efficacy of a *Leishmania* amastigote-specific protein, member of the super-oxygenase family, against visceral leishmaniasis. *PLoS Negl Trop Dis.* 7;e2148, 2013]. Dessa forma, procura-se a associação de que, nos animais considerados como protegidos, que os mesmos apresentem baixa carga parasitária em relação aos controles do experimento e que seus esplenócitos produzam e secretem níveis elevados de IFN-gama e IL-12, e baixos níveis de IL-4 e IL-10, após a estimulação *in vitro* dos mesmos.

Desta forma, antígenos que são capazes de estimular o desenvolvimento de uma resposta tipo Th1, destacada pela produção de citocinas como IFN-gama em esplenócitos de camundongos infectados com *Leishmania*, podem-se apresentar como pré-candidatos vacinais promissores para utilização como vacinas efetivas contra esse parasita.

Foram encontrados, no estado da técnica, alguns documentos de patente que tratam do uso de proteínas e peptídeos no imunodiagnóstico da leishmaniose e como vacina contra essa doença, como por exemplo, o documento PI0804859-2, intitulado "Peptídeos sintéticos para a obtenção de polímero protéico para imunização contra Lesihmaniose, produtos e seus usos", que se refere a dois peptídeos sintéticos antigênicos (epitopos/mimotopos), selecionados e sintetizados pelas técnicas de *phage display* e spot synthesis e ao processo de obtenção de polímero protéico obtido através da conjugação destes peptídeos. O antígeno polimérico formado por

esses peptídeos foi capaz de induzir proteção contra Leishmaniose Visceral e oferecer diagnóstico específico para sua detecção.

O documento US5834592, intitulado "Leishmania antigens for use in the therapy and diagnosis of Leishmaniasis" , descreve polipeptídeos que contêm pelo menos uma porção imunogênica de um ou mais antígenos de Leishmania e o uso desses polipeptídeos na prevenção, tratamento e diagnóstico de leishmaniose.

O documento US5674503, intitulado "Peptides capable of eliciting an immune response to leishmaniasis and methods of using the same", descreve peptídeos capazes de induzir uma resposta imune contra leishmaniose.

O documento WO2012/019268, intitulado "An immunogenic composition for use in a vaccine and an immunodiagnostic test kit for visceral leishmaniasis", descreve uma vacina e kit imunodiagnóstico para leishmaniose, desenvolvidos através da identificação, produção e seleção de novos antígenos por meio de análise proteômica, bioinformática, síntese peptídica e imunoensaios enzimáticos.

No entanto, nenhum dos peptídeos apresentados nos documentos de patente encontrados nas buscas é semelhante aos da presente tecnologia. Além do mais, as vacinas comerciais disponíveis contra a LVC no Brasil podem induzir a produção de níveis elevados de anticorpos específicos aos parasitas nos animais vacinados, assim, tais animais acabam não sendo diferenciados daqueles que realmente se encontram infectados por *Leishmania* nas triagens sorológicas realizadas pelos órgãos de Saúde competentes. Em paralelo, os antígenos vacinais apresentam eficácia moderada e possuem custos de produção e venda elevados, o que acaba por limitar seu acesso à maioria da população.

Os antígenos atualmente utilizados como vacinas contra as leishmanioses são proteínas completas, as quais contêm regiões comuns a proteínas de outras doenças, o que pode gerar reações cruzadas nos animais vacinados. Também, tais proteínas podem conter epitopos (peptídeos) que apresentam efeito benéfico, mas outros que apresentam efeito nocivo nos

animais imunizados, comprometendo, dessa forma, a eficácia completa da vacina.

A presente tecnologia descreve uma composição vacinal para prevenção e/ou tratamento da Leishmaniose visceral, utilizando como antígenos, peptídeos expressos na superfície externa de bacteriófagos não-nocivos ao hospedeiro mamífero, ou sintetizados, sendo utilizados isolados ou associados. Esses peptídeos foram selecionados pela técnica *phage display* e induziram resposta imune específica do tipo Th1, destacada pela maior produção de IFN-gama.

Os pontos inovadores da presente baseiam-se na identificação de marcadores inéditos com aplicação no desenvolvimento de vacinas contra as leishmanioses, utilizando como antígenos, peptídeos expressos na superfície externa de bacteriófagos não-nocivos ao hospedeiro mamífero, ou na sua forma sintética de polímeros peptídicos isolados ou associados.

O uso de pequenos antígenos na forma de peptídeos, como apresentado no presente pedido de patente, tende a permitir uma vacina mais específica, de modo que o animal imunizado poderá desenvolver uma resposta imune frente apenas a tal antígeno, específica a ele e à doença para a qual o antígeno foi selecionado. Tais antígenos apresentam também uma produção técnica mais simples, rápida e de menor custo do que a obtenção de proteínas recombinantes completas; têm maior estabilidade pós-produção e podem ser disponibilizados mais facilmente para a grande maioria da população.

DESCRIÇÃO DETALHADA DA TECNOLOGIA

A presente tecnologia compreende novas composições vacinais empregando peptídeos selecionados por *phage display*, incorporados aos clones de bacteriófagos ou na sua forma sintética, para que, isoladamente ou em associação, sejam aplicados na composição de vacinas preventivas e/ou terapêuticas contra as leishmanioses. A composição vacinal aqui descrita pode ser administrada pelas vias dérmica, intramuscular, intravenosa, intraperitoneal, subcutânea, transdérmica ou como dispositivos que possam ser implantados ou injetados.

Para o desenvolvimento da presente tecnologia inicialmente, um processo de seleção negativa por *phage display* foi utilizado. O mesmo baseou-se na exposição de uma biblioteca recombinante de clones de fagos de sete resíduos de aminoácidos frente às microesferas incorporadas com moléculas de IgGs purificadas de amostras de soros de cães sadios. Os clones que não se ligaram a tais microesferas incorporadas com as IgGs, após 3 ciclos de seleção, foram recuperados e submetidos a um processo de seleção positiva, no qual os mesmos foram expostos para microesferas incorporadas às moléculas de IgGs purificadas de soros de cães com LV. Aqueles clones que se mantiveram aderidos a essas microesferas foram recuperados, amplificados e sequenciados. Tais clones foram utilizados em experimentos de estimulação *in vitro* de esplenócitos de camundongos BALB/c cronicamente infectados com *L. infantum*, sendo então realizada a dosagem das citocinas IFN-gama, IL-4 e IL-10, após os estímulos individuais específicos dos clones, sendo que foram selecionados apenas aqueles clones cuja estimulação tenha induzido a produção de níveis elevados de IFN-gama e baixos níveis de IL-4 e IL-10.

Parasitas

A amostra MHOM/BR/1970/BH46 de *L. infantum* foi utilizada. Os parasitas foram cultivados em meio Schneider's completo, o qual foi constituído pelo meio Scheneider's (SIGMA) acrescido com 20 % de soro fetal bovino (SIGMA) inativado, 20 miliMolar (mM) de L-glutamina, 50 microgramas por mililitro (µg/mL) de gentamicina, 200 unidades por mL de penicilina e 100 µg/mL de estreptomicina, em pH 7,4 e a 25°C.

Infecção desafio

Para a realização da infecção experimental nos animais, $1x10^7$ formas promastigotas em fase estacionária de crescimento de *L. infantum* foram inoculadas no coxim plantar direito de 8 animais. Os camundongos foram monitorados durante 8 semanas após o desafio, quando foi realizada a eutanásia dos animais para os experimentos de imunogenicidade e determinação da carga parasitária. A carga parasitária foi realizada a fim de atestar que os animais estavam realmente infectados pelo parasita.

A presente tecnologia poderá ser melhor compreendida a partir dos exemplos, não limitantes, que seguem:

Exemplo 1- Amostras de soros e purificação de anticorpos da classe IgG

Amostras de soros de cães com LV (n=20) e de cães sadios (n=40) foram utilizadas para a purificação de imunoglobulinas da classe IgG. Tal procedimento foi realizado por meio do acoplamento dos pools de soros em microesferas magnéticas (*beads* magnéticos) conjugadas à proteína G (Dynabeads, Invitrogen). Para tal, $2x10^9$ partículas de microesferas foram lavadas por 3 vezes em tampão MES 0.1 M pH 5.0 e foi adicionado às mesmas 375 μL do pool de soros de cães sadios, para um volume final de 500 μL ou 300 μL do pool de soros de cães com LV, para um volume final de 500 μL. A proporção de anticorpos para a quantidade das microesferas em todos os casos foi de 1:1. Em seguida, foi realizada uma incubação por 40 min, sob agitação constante, à temperatura ambiente (T.A). As microesferas adsorvidas com os anticorpos foram lavadas 3 vezes em tampão MES 0.1 M pH 5.0, com a finalidade de se retirar os anticorpos IgGs não-aderidos. Para a finalização da ligação covalente entre as microesferas e os anticorpos, o sistema "*beads*-anticorpo" foi lavado 2 vezes com 1 mL de tampão trietanolamina 0.2M pH 8.2 e ressuspendido em 1 mL de tampão de ligação covalente (20 mM de dimetilpimelinidato/HCl em tampão trietanolamina) por 30 min, sob agitação constante e à T.A. A neutralização da reação foi feita pela incubação do sistema com 1 mL de tampão Tris 50 mM pH 7.5, por 15 min e a T.A. Em seguida, as microesferas incorporadas foram lavadas por 3 vezes em tampão TBS-T 0.1% de Tween 20 e bloqueadas com solução de bloqueio (5% de BSA em TBS-T 0.05% de Tween 20) por 1 h e a 37°C, sendo ressuspendidas em 200 μL de tampão TBS. Para certificação do acoplamento, 5 μL dos *beads* cobertos pelas IgGs foram incubados por 1 h e a 37°C com o anticorpo anti-IgG de cão (diluição de 1:5.000). Após a incubação, os *beads* foram lavados 3 vezes com TBS-Tween 0.1% e a reação foi revelada pela adição do substrato tetrametilbenzidina (TMB). A reação foi então interrompida pela adição de ácido

sulfúrico 2 N e a leitura da absorbância foi efetuada a 450 nm, em leitor de microplacas (Titertek Multiskan Plus, Flow Laboratories, USA).

Exemplo 2- Ciclos de bio-panning

Para a realização das seleções negativa e positiva nos ciclos de bio-pannings, $1x10^{11}$ partículas virais de uma biblioteca recombinante contendo peptídeos fusionados de sete resíduos de aminoácidos expressos em fagos filamentosos (biblioteca Ph.D.-C7C, New England, BioLabs®) foram utilizadas. As mesmas foram diluídas em 190 μL de TBS-Tween 0.1% para a seleção dos ligantes nas microesferas incorporadas pelos anticorpos IgGs.

O processo foi iniciado pela seleção negativa. Para tal, a biblioteca de bacteriófagos recombinantes foi incubada por 30 min e a T.A com as microesferas acopladas com as IgGs dos cães sadios e, em seguida, as microesferas foram precipitadas por atração magnética ao suporte Dynal Biotech (no. 12020), sendo o sobrenadante, contendo os clones que não se aderiram às IgGs desses animais; transferido para um novo tubo. Tal procedimento foi realizado por 3 vezes em sequência. Apenas os clones de fagos que não se aderiram à tais anticorpos foram recuperados.

Para a seleção positiva, o sobrenadante dos fagos recuperados foi transferido para um novo tubo contendo as microesferas acopladas com as IgGs de cães com LV, sendo incubadas por 30 min e à T.A. As microesferas contendo o sistema *beads*-anticorpos precipitado nos tubos dos bio-panning das IgGs dos cães, mais os fagos de interesse aderidos, foram lavadas por 10 vezes com TBS-Tween 20 0.1% e os fagos foram removidos da ligação pela adição de 500 μL de tampão glicina 0.2 M pH 2.0, sendo tal solução neutralizada pela adição de 75 μL de Tris-base pH 9.0. As microesferas contendo o sistema *beads*-anticorpos precipitado nos tubos dos bio-panning das IgGs de cães com LV, contendo os fagos de interesse aderidos, foram processados e titulados.

Exemplo 3- Titulações dos clones de fagos

Ao final do 3º ciclo de bio-panning, os clones de fagos recuperados ao final do 1º, 2º e 3º ciclos foram titulados. Para tal, os mesmos foram submetidos a diluições seriadas exponenciais (log^{10}) em meio LB líquido com

ampicilina (100 mg/mL), sendo as diluições de 10^0 até 10^{-6} para os eluatos não amplificados e de 10^{-6} a 10^{-12} para os eluatos amplificados. A cada diluição, 200 µL da cultura de *E. coli* ER2738 na fase *mid-log* (OD_{600nm}~0,5) foram acrescentados. Após a infecção das bactérias pelos fagos, a cultura foi transferida para novos tubos contendo 3 mL de ágar Top (10 g de Bacto-Triptona, 5 g de extrato de levedura, 5 g de NaCl, 7 g de agarose, 1 g de MgCl2.6H2O, por 1 L de água deionizada) e espalhadas em placa de Petri contendo meio LB sólido acrescido de IPTG (0.5 mM), Xgal (40 µg/mL) e ampicilina. Para cada titulação, uma placa de cultura foi confeccionada e as mesmas foram incubadas à 37°C por 16 h. Após, as colônias azuis e individualizadas foram quantificadas. Para estimar os valores dos títulos, o número total de colônias contadas foi multiplicado pelo fator de diluição de cada placa.

Exemplo 4- Amplificação e extração do DNA dos fagos

As colônias de cor azul e individualizadas, presentes nas placas de cultura obtidas quando da titulação do 3° ciclo de bio-panning, foram coletadas para a extração de DNA. Para a amplificação dos fagos coletados, 1.2 mL da cultura de células *E. coli* ER2738 em fase *early-log* (OD_{600nm}~0,3) foram distribuídos em cada poço de uma placa "deepwell". Com palitos de dente estéreis, 96 colônias azuis foram retiradas da placa de Petri e transferidas para uma placa de cultura de 96 poços individualizados. A placa foi vedada e incubada por 5 h, sob agitação constante, a 37°C. Após a incubação, a placa foi centrifugada por 20 min a 2.250 x *g*, sendo o sobrenadante transferindo para outra placa. À essa 2ª placa, foi adicionado PEG/NaCl (1/6 do volume total do sobrenadante), sendo a mesma incubada por 14 h e a 4°C. Após, a placa foi centrifugada por 1 h, o sobrenadante foi retirado e o pellet foi ressuspendido em tampão iodeto (10 mM de Tris HCl pH 8.0, 1 mM de EDTA e 4 M de NaI). As placas foram agitadas vigorosamente e, em seguida, adicionou-se etanol absoluto. Após uma incubação de 10 min, as placas foram centrifugadas (2.250 x *g* a 4°C e por 10 min), sendo o sobrenadante descartado. O precipitado contendo o DNA foi lavado com 500 µL de etanol 70% e novamente centrifugado. Finalmente, o DNA foi diluído em 20 µL de água milli-Q e sua

qualidade foi avaliada após uma eletroforese em gel de agarose 0.8%, corado com brometo de etídio (10 µg/mL).

Exemplo 5- Sequenciamento dos clones de fagos

A reação de sequenciamento utilizando o DNA dos fagos de interesse foi realizada com 500 ng de DNA de cada fago selecionado, 5 pmol do *Primer* 96 gIII (5'-OH CCC TCA TAG TTA GCG TAA CG-3'-Biolabs) e o *pre-mix* (DYEnamic ET Dye Terminator Cycle Kit-Amersham Biosciences). A reação de 35 ciclos ocorreu em um termociclador de placas, nas seguintes condições: desnaturação a 95°C por 20 seg, anelamento a 58°C por 15 seg e extensão a 60°C por 60 seg. Os *amplicons* gerados da reação de sequenciamento foram precipitados com 1 µL de acetato de amônio e 27.5 µL de etanol absoluto. A placa foi centrifugada por 45 min a 2.432 x *g* e o sobrenadante foi descartado. Adicionou-se 150 µL de etanol 70% ao pellet e o material foi centrifugando novamente por 10 min e a 2.432 x *g*, sendo o sobrenadante descartado. A placa permaneceu invertida sobre um papel toalha e foi então centrifugada a 486 x *g*, por 1 min. Em seguida, a placa foi coberta com papel alumínio, permanecendo por 5 min para a evaporação completa do etanol remanescente. O precipitado foi ressuspendido em tampão de diluição (DYEnamic ET Dye Terminator Cycle Kit-Amersham Biosciences) e o sequenciamento foi realizado no sequenciador automático MegaBace 1000 (Amersham Biosciences).

A dedução *in silico* das sequências de aminoácidos do DNA dos fagos de interesse, para a identificação dos peptídeos exógenos, foi realizada pelo programa DNA2PRO12, que é designado para a dedução de sequências de insertos das bibliotecas da *New England Biolabs* (Ph.D.-C7CTM), como de outras bibliotecas de interesse que contenham as sequências inicial e final do vetor. O programa automaticamente localiza a posição do inserto, traduz o mesmo e indica os possíveis erros na tradução (tais como códons inesperados ou erros na sequência próxima). As sequências que não puderam ser traduzidas pelo programa foram analisadas manualmente.

Exemplo 6- Avaliação da carga parasitária

Com a finalidade de verificar que os animais infectados com *L. infantum* apresentavam o parasita em seus órgãos internos, animais (n=8) foram infectados subcutaneamente com essa espécie do parasita e, 8 semanas após, os mesmos foram eutanasiados para a coleta de órgãos e as avaliações parasitológica e imunológica. Na análise dos resultados, observou-se que em todos os órgãos avaliados os parasitas foram encontrados, porém, os mesmos estavam mais presentes no baço e linfonodo drenante dos animais (Tabela 1). Dessa forma, o baço foi considerado como um sítio sistêmico de presença dos parasitas e sua coleta para as análises de imunogenicidade dos clones de fagos selecionados tornaram-se, dessa forma, factíveis de serem realizadas.

Tabela 1- Carga parasitária de animais infectados com *L. infantum*.

Órgão avaliado	**Média ± desvio-padrão do log do número de parasitas obtido pela técnica de diluição limitante**
Fígado	3,8±0,5
Baço	6,5±0,6
Medula óssea	4,5±0,7
Linfonodo drenante	6,8±0,8

Os resultados correspondem à média ± desvio-padrão dos grupos, em 3 experimentos realizados e que apresentaram resultados similares.

Após a verificação da infecção sistêmica nos animais, o baço dos mesmos foi utilizado para a cultura dos esplenócitos e os estímulos individuais utilizando os clones de fagos selecionados nos 3 ciclos de bio-pannings. Dos 96 clones que foram coletados após o 3º ciclo de bio-panning de seleção positiva, 21 deles foram selecionados, devido à perda das sequências de outros clones e à presença de sequências repetidas dos peptídeos entre os diferentes clones. Na tabela 2, são mostradas as sequências dos peptídeos de interesse que foram identificadas nos 21 clones pré-selecionados.

Tabela 2- Sequências dos clones de fagos expressando os peptídeos de interesse que foram selecionados após os 3 ciclos de bio-pannings de seleção positiva.

Clones	Identidade das sequências de aminoácidos dos peptídeos de interesse
A10	L L S S K T L
B10	L S F P F P G
B11	H L S V L H A
C1	F T S F S P Y
C2	Q A T H F H S
D9	L A S L P F R
E3	T H V F S W I
E10	A C D P S P N
E11	T P S L H R S
F1	T A Met A R S A
F3	V A L L P H H
F4	Q S P P A L L
F8	F S L L G S L
F9	V L L G P F P
F11	Y P F S L L H
G2	S L G P Q I K
G5	Met S P T Y L L
G9	D R A A L S L
G11	A L T P Q L L
G12	Q T S P P L A
H5	F P L F G L S

Exemplo 7- Imunogenicidade dos clones de fagos selecionados

Posteriormente, os 21 clones pré-selecionados foram utilizados na estimulação dos esplenócitos dos animais infectados e posterior dosagem de citocinas pró e anti-inflamatórias. Os resultados das dosagens de IFN-gama, IL-4 e IL-10 são mostrados na tabela 3, em valores de absorbância. Os valores apresentados são representativos de 3 experimentos realizados de maneira independente e que apresentaram resultados similares. Um clone de fago silvestre, não expressando nenhum peptídeo exógeno (fago selvagem) e um clone de fago expressando um peptídeo não-relacionado com sequências

primárias de proteínas de *Leishmania* (fago aleatório), foram utilizados como controles.

Tabela 3- Resultados das médias e desvios-padrão das dosagens de citocinas utilizando os clones dos fagos selecionados.

Clone	Citocinas		
	IFN-gama	*IL-4*	*IL-10*
A10	0,138	0,128	0,132
B10	0,150	0,128	0,131
B11	0,143	0,124	0,163
C1	0,145	0,121	0,127
C2	0,148	0,129	0,135
D9	0,146	0,117	0,126
E3	0,143	0,127	0,206
E10	0,136	0,123	0,127
E11	0,134	0,126	0,128
F1	0,152	0,126	0,129
F3	0,130	0,129	0,134
F4	0,128	0,116	0,141
F8	0,116	0,128	0,126
F9	0,178	0,127	0,137
F11	0,138	0,120	0,132
G2	0,125	0,128	0,137
G5	0,152	0,130	0,135
G9	0,149	0,133	0,138
G11	0,134	0,124	0,120
G12	0,133	0,125	0,133
H5	0,130	0,134	0,126
Meio	0,119	0,126	0,127
Selvagem	0,110	0,145	0,128
Aleatório	0,140	0,143	0,145

Utilizando as absorbâncias detectadas e as curvas-padrão de cada citocina, os seguintes níveis de produção de IFN-gama, IL-4 e IL-10 foram obtidos, em unidades de picogramas por mililitro (tabela 4).

Tabela 4- Níveis de produção de IFN-gama, IL-4 e IL-10 (em pg/mL)

Clone	Citocinas (em pg/mL)		
	IFN-gama	*IL-4*	*IL-10*
A10	194	110	110
B10	222	110	108
B11	202	101	180
C1	207	92	98
C2	216	113	117
D9	210	82	96
E3	202	107	246
E10	192	99	97
E11	190	105	101
F1	225	105	104
F3	178	113	115
F4	172	80	130
F8	102	110	96
F9	328	107	120
F11	194	90	110
G2	166	110	120
G5	245	118	117
G9	218	125	122
G11	190	101	88
G12	188	103	112
H5	178	128	96
Selvagem	125	121	101
Aleatório	144	139	165

Exemplo 8- Razão entre os níveis das citocinas

Após a obtenção dos resultados relativos às concentrações das citocinas, optamos por calcular a proporção entre IFN-gama/IL-4 e IFN-gama/IL-10, no sentido de selecionarmos aqueles clones cujos peptídeos de interesse tenham sido capazes de produzir maiores níveis de IFN-gama em relação aos níveis de IL-4 e IL-10. Os dados são apresentados na tabela 5.

Tabela 5- Proporção entre os níveis das citocinas IFN-gama/IL-4 e IFN-gama/IL-10 nos sobrenadantes das culturas estimuladas com os clones de fagos selecionados.

Clone	Citocinas (razão)	
	IFN-gama/IL-4	*IFN-gama/IL-10*
A10	1,76	1,76
B10	2,02	2,06
B11	2	1,12
C1	2,25	2,11
C2	1,91	1,85
D9	2,56	2,19
E3	1,89	0,82
E10	1,94	1,98
E11	1,81	1,88
F1	2,14	2,16
F3	1,58	1,55
F4	2,15	1,32
F8	0,93	1,06
F9	3,07	2,73
F11	2,16	1,76
G2	1,51	1,38
G5	2,08	2,09
G9	1,74	1,79
G11	1,88	2,16
G12	1,83	1,68
H5	1,39	1,85
Selvagem	1,03	1,24
Aleatório	1,04	0,87

Na análise dos resultados, observou-se que houve uma diferença interna na produção das citocinas IFN-gama, IL-4 e IL-10 entre os clones selecionados. Os controles apresentaram proporção de IFN-gama com IL-4 ou IL-10 próximos de 1.0. Aqueles clones cuja produção de IFN-gama foi, pelo menos, 2 vezes maior que a produção de IL-4 e IL-10, apresentam-se como candidatos para serem empregados como vacina contra as leishmanioses, haja vista que, após o estímulo de esplenócitos de camundongos com LV, os mesmos induziram a uma maior produção de citocinas do tipo Th1, em relação a produção das citocinas Th2.

Deste feita, os clones **B10, C1, D9, F1, F9** e **G5** (Seq ID Nº 1 a 6) apresentaram-se como os melhores candidatos vacinais e serão empregados em testes experimentais *in vivo* para avaliação da proteção de camundongos BALB/c contra a infecção experimental com *L. infantum*.

REIVINDICAÇÕES

1- **Composição vacinal contra leishmaniose visceral canina caracterizada por compreender** pelo menos um dos peptídeos de Seq ID Nº 1 a 6, expressos na superfície externa de bacteriófagos ou sintetizados e excipientes farmacologicamente e farmaceuticamente aceitáveis.

2- **Composição vacinal contra leishmaniose visceral canina, de acordo com a reivindicação 1, caracterizada por** ser administrada pelas vias dérmica, intramuscular, intravenosa, intraperitoneal, subcutânea, transdérmica ou como dispositivos que possam ser implantados ou injetados.

3- **Peptídeos sintéticos caracterizados** por compreender as sequências representadas por SEQ ID Nº 1 a 6.

4- **Uso dos peptídeos sintéticos definidos na reivindicação 3, caracterizado** por ser na prepraração de composições vacinais contra leishmaniose.

5- **Uso da composição vacinal definida na reivindicação 1, caracterizado** por ser no tratamento e prevenção da leishmaniose visceral canina.

RESUMO

COMPOSIÇÃO VACINAL CONTRA LEISHMANIOSE VISCERAL CANINA, PEPTÍDEOS SINTÉTICOS E USO

A presente tecnologia descreve uma composição vacinal para prevenção e/ou tratamento da leishmaniose visceral canina, utilizando como antígenos, peptídeos expressos na superfície externa de bacteriófagos não-nocivos ao hospedeiro mamífero, ou sintetizados, sendo utilizados isolados ou associados. A tecnologia descreve também o uso desses peptídeos na preparação de das composições vacinais contra leishmaniose visceral. Esses peptídeos foram selecionados pela técnica *phage display* e induzem resposta imune específica do tipo Th1, destacada pela maior produção de IFN-gama.

Sensitivity and Specificity of an Enzyme-linked Immunosorbent Assay for the Detection of Infectious Bovine Rhinotracheitis Viral Antibody in Cattle

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ABSTRACT

An enzyme-linked immunosorbent assay was developed to detect bovine serum antibody to infectious bovine rhinotracheitis virus. The specificity of this assay in 304 bovine sera, collected from an infectious bovine rhinotracheitis virus-free herd, was 100%; in sera from 62 cattle inoculated with an intranasal vaccine, its diagnostic sensitivity was 27.4% at one month and 100% at six months, postvaccination. In 303 bovine sera with standard serum neutralizing antibody titers of $\geqslant$1:2 it showed 100% sensitivity; and in 463 random diagnostic samples, comparative tests indicated that enzyme-linked immunosorbent assay detected more seropositive animals (61.6%) than the standard serum neutralizing test (49.9%). The enzyme-linked immunosorbent assay method was considered to be technically superior as a routine diagnostic test for the detection of infectious bovine rhinotracheitis viral antibody in bovine sera.

Key words: Infectious bovine rhinotracheitis, enzyme-linked immunosorbent assay.

RÉSUMÉ

Cette expérience consistait à utiliser la technique immunoenzymatique ELISA pour rechercher les anticorps contre le virus de la rhino-trachéite infectieuse bovine, dans le sérum de bovins. Elle s'avéra efficace à 100%, pour la détection des anticorps précités, dans 304 échantillons de sérum qui provenaient des sujets d'un troupeau exempt du virus de la rhino-trachéite infectieuse bovine. Son efficacité n'atteignit que 27,4%, lors de l'épreuve du sérum de 62 bovins, un mois après leur vaccination intranasale; six mois après cette vaccination, son efficacité atteignit cependant 100%. Elle démontra aussi une efficacité totale, lors de l'épreuve de 303 échantillons de sérum bovin dont le titre d'anticorps égalait ou dépassait 1:2 avec l'épreuve standard de séroneutralisation. Lors de l'épreuve de 463 échantillons de sérum choisis au hasard parmi ceux qui avaient été soumis pour diagnostic, la technique immunoenzymatique ELISA décela 61,6% des sujets séropositifs, comparativement à 49,9% pour l'épreuve standard de séroneutralisation. De tels résultats amenèrent les auteurs à admettre la supériorité de la technique ELISA comme épreuve routinière de diagnostic pour la recherche des anticorps contre le virus de la rhino-trachéite infectieuse bovine, dans le sérum de bovins.

Mots clés: rhino-trachéite infectieuse bovine, technique immunoenzymatique ELISA.

INTRODUCTION

The serum neutralization (SN) test is routinely used by Agriculture Canada for serodiagnosis of infectious bovine rhinotracheitis (IBR) virus infection in cattle for export certification purposes. The SN test is specific in terms of yielding negative results on samples collected from known noninfected animals, however, the sensitivity of this test is considered to be suboptimal and consequently may give false negative reactions. In addition the SN test requires expensive tissue culture systems and is time consuming. Recently, several reports on enzyme-linked immunosorbent assay (ELISA) for the detection of IBR viral antibodies have been published (1-3). This communication describes the application of an ELISA for the detection of IBR viral antibodies in bovine sera and measures the performance of the test in terms of its diagnostic specificity and sensitivity. The feasibility of adopting an ELISA for routine serodiagnosis of IBR virus is also discussed.

MATERIALS AND METHODS

SERA

Three hundred and four sera which had been collected from the Animal Diseases Research Institute (ADRI) Lethbridge cattle herd were used to determine the diagnostic specificity of the ELISA. With the exception of the occasional introduction of an IBR seronegative bull this has been a closed herd for seven years and no clinical evidence of IBR virus infection, based on repeated SN tests, has been observed during this period. The last outside animal was introduced 18 months ago and all animals in the herd have been IBR seronegative for four consecutive biannual tests. For the diagnostic sensitivity assay, 62 ten month old calves from another institutional herd were inoculated with a modified live intranasal IBR vaccine (Connaught Laboratories, Willowdale, Ontario) and serum samples were collected at one and six months postvaccination. In order to assess relative sensitivity, 303 sera, which had standard SN antibody at serum dilutions of

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1:2 or greater, were selected. Finally to compare ELISA with the standard and modified SN tests, 121 sera from an IBR virus-infected herd and 342 sera from animals of unknown infection status which had been submitted for diagnostic purposes, were used. A positive reference serum was prepared by pooling sera collected from ten IBR virus-infected cattle with demonstrable SN antibody, and a negative reference serum was obtained by pooling sera from five cattle in the noninfected herd.

SERUM NEUTRALIZATION TESTS

Standard SN and modified SN tests (4,5) were performed in a microsystem. Twofold serial dilutions (1:2-1:256) of heat inactivated test serum (56° C, 30 min) 0.05 mL were mixed with equal volumes of IBR virus, Colorado strain, containing 100 $TCID_{50}$. The mixtures were incubated for 1 h at room temperature for the standard SN and for 24 h at 37° C in a CO_2 incubator for modified SN. In the modified SN a final concentration of 5% guinea pig complement was added to the serum dilution. At the end of incubation, 0.05 mL of secondary bovine fetal kidney cells were added to the test wells and the microplates were further incubated for three days at 37° C in a CO_2 incubator. The highest dilution of serum which gave complete neutralization of the virus was recorded as the SN titer.

ELISA ANTIGEN PREPARATION

In a preliminary study three different methods of antigen preparation reported previously (1-3) were assessed. A Freon 113 (DuPont, Calgary, Alberta) purified antigen similar to that described by Herring *et al* (2) proved satisfactory and gave consistent results. Briefly, tissue culture fluid collected from Colorado strain or 108 strain of IBR virus-infected secondary bovine kidney cells at maximum cytopathic activity was clarified by centrifugation at 1000 g for 10 min. The supernatant was decanted and concentrated by ultracentrifugation at 40,000 g for 1 h. The pellets were resuspended to 1/50 of their original volume in NTE buffer (0.15M NaCl, 0.01M tris, 1 mM EDTA, pH 7.2). The virus suspension was treated with equal volume of Freon 113 for 2 min and the aqueous phase collected after centrifugation at 2000 x g for 10 min and used as the source of antigen. The concentration of the antigen was determined by titration with 1:100 dilutions of the reference positive and negative sera. The antigen dilution, which gave absorbance at 492 nm (A_{492}) $\geqslant$ 1.200 with a reference positive serum, was used as the working dilution of the antigen.

ELISA PROCEDURE

Antigen Coating — Linbro EIA microtitration plates with 96 flat bottom wells (Flow Laboratories, Hamden, Connecticut; Cat. No. 76-381-04) were used for the test. The working dilution of the antigen was made in 0.05M carbonate buffer with 0.125M NaCl at pH 9.5. The wells of the microtitration plates, columns 2 to 11 inclusive, were sensitized by adding 0.2 mL of the diluted antigen per well. The wells in columns 1 and 12 received 0.2 mL of the carbonate buffer and were used as various controls.

After incubation at 4° C overnight in a covered container, the wells were decanted, dried in air, wrapped in a plastic bag and stored at -63° C until used.

Test Sera — ELISA tests were performed in triplicate plates as shown in Figure 1. To eliminate nonspecific hydrophobic binding to the plate surface, 0.35 mL diluent [Dulbecco PBS, containing 0.5% Tween 20 and 1% heat inactivated (56° C 30 min) horse serum] was added to each well and incubated 10-15 min at room temperature, followed by three washings with tap water in a Titertek Microplate Washer (Flow Laboratories, Hamden, Connecticut). Sera were diluted 1:100 in the same diluent. Appropriate wells received 0.2 mL of the diluted sera after which the plates were incubated for 2 h at 37° C and then washed five times as above.

Peroxidase Conjugate — Optimum concentration of peroxidase-conjugated IgG fraction of rabbit antibovine IgG (heavy and light chains) was diluted in the diluent (e.g. 1:16,000 dilution with Lot No. 16668 conjugate, Cappel Laboratories, Cochranville, Pennsylvania). The conjugate, (0.2 mL per well) was allowed to react for 1 h at 37° C, followed by washing three times in the microplate washer. An optimum concentration of the conjugate is defined as the highest dilution of the conjugate which gave the highest positive/negative (P/N) ratio and gave an A_{492} value of $\geqslant$ 1.200 with a reference positive serum.

Substrate — Freshly prepared o-phenylenediamine, 1 mg/mL in 0.1M citrate buffer pH 5.5 with 3 mM H_2O_2 was used as the substrate. After the substrate (0.2 mL per well) had been incubated for 45 min at room temperature, 50 μL *of* 4*N* H_2SO_4 were added to each well to stop the reaction.

INTERPRETATION

The A_{492} value of each plate was read with a Titertek Multiskan Pho-

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	St	1	2	3	4	AC	33	34	35	36	AC	CC
B	St	5	6	7	8	R-	37	38	39	40	R+	S-
C	St	9	10	11	12	R-	41	42	43	44	R-	S-
D	St	13	14	15	16	R+	45	46	47	48	R-	S-
E	St	17	18	19	20	R-	49	50	51	52	R+	S+
F	St	21	22	23	24	R-	53	54	55	56	R-	S+
G	St	25	26	27	28	R+	57	58	59	60	R-	S+
H	St	29	30	31	32	AC	61	62	63	64	AC	CC

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	St	49	50	51	52	AC	17	18	19	20	AC	CC
B	St	53	54	55	56	R-	21	22	23	24	R+	S-
C	St	57	58	59	60	R-	25	26	27	28	R-	S-
D	St	61	62	63	64	R+	29	30	31	32	R-	S-
E	St	33	34	35	36	R-	1	2	3	4	R+	S+
F	St	37	38	39	40	R-	5	6	7	8	R-	S+
G	St	41	42	43	44	R+	9	10	11	12	R-	S+
H	St	45	46	47	48	AC	13	14	15	16	AC	CC

Plate 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	St	21	22	23	24	AC	53	54	55	56	AC	CC
B	St	25	26	27	28	R-	57	58	59	60	R+	S-
C	St	29	30	31	32	R-	61	62	63	64	R-	S-
D	St	17	18	19	20	R+	49	50	51	52	R-	S-
E	St	13	14	15	16	R-	45	46	47	48	R+	S+
F	St	1	2	3	4	R-	33	34	35	36	R-	S+
G	St	5	6	7	8	R+	37	38	39	40	R-	S+
H	St	9	10	11	12	AC	41	42	43	44	AC	CC

Fig. 1. Diagram of ELISA test pattern on EIA Microtiter plates.
Numbers: test samples
St: substrate control
AC: antigen control
CC: conjugate control
R+, R-: reference positive and negative serum
S+, S-: reference positive and negative serum control.

tometer (Flow Laboratories). A P/N ratio was calculated by dividing the A_{492} value of each test sample replicate with the average A_{492} of reference negative sera for that plate and from the triplicate test an average P/N ratio was determined. The results were interpreted as follows:

P/N ratio <1.50
negative
P/N ratio $\geqslant 1.50 \sim < 2.00$
suspicious
P/N ratio $\geqslant 2.00$
positive

The performance of the ELISA was considered in terms of the following parameters as defined by Vecchio (6).

$$\text{Specificity (\%)} = \frac{\text{No. of ELISA negative samples}}{\text{all negative samples}} \times 100$$

$$\text{Sensitivity (\%)} = \frac{\text{No. of ELISA positive samples}}{\text{all positive samples}} \times 100$$

Repeat of Test — Infrequent unusual A_{492} values were not included in the calculation unless more than one of the triplicate tests was affected, in which case the sample was retested. The entire test was repeated if the A_{492} values of the reference sera deviated from expected values.

STATISTICAL ANALYSIS

Statistical tests included analysis of variance of the P/N ratios by randomized block design, and correlation coefficients between tests, by Pearson product-moment method (7).

RESULTS

ELISA ANTIGEN

Titration of three lots of Freon 113-purified IBR viral ELISA antigen is shown in Figure 2. With positive reference serum, higher concentrations of antigen gave progressively higher A_{492} values and higher P/N ratios over an antigen dilution range of 1:40 to 1:5120. At antigen dilutions of 1:10 and 1:20, plateau reactions were observed. With negative reference serum, the antigen dilutions did not affect either the A_{492} values or the P/N ratios. Both the Colorado strain and 108 strain of IBR virus, which both belong to restriction-endonuclease type 1A (8), produced satisfactory ELISA antigen. A group of negative (n = 162) and positive (n = 30) sera were tested with antigen dilutions of 1:50 and 1:300 (Table I). The correlation coefficients between the antigen dilutions for the negative and positive sera were 0.506 and 0.927, respectively ($P < 0.001$). Analysis of variance indicated that P/N ratios were significantly different between the dilutions at the 5% level with the negative sera and at the 0.1% level with the positive sera.

TABLE I. Effect of Antigen Concentration on Positive/Negative Ratio

Sera	Antigen dilution	Positive/Negative ratio[a] Mean ± S.E.	Minimum	Maximum	Correlation[b] coefficient
Negative	1:300	1.024 ± 0.009	0.610	1.460	
(N = 162)	1:50	1.002 ± 0.009	0.760	1.440	0.506
Positive	1:300	4.649 ± 0.240	2.870	7.440	
(N = 30)	1:50	5.785 ± 0.432	3.140	11.710	0.927

[a]Analysis of variance of P/N ratios are significant at 5% level with negative sera and 0.1% level with positive sera
[b]All correlation coefficients are significant at 0.1% level

TABLE II. Specificity Test of the ELISA Using Bovine Sera Collected from an IBR Virus Free Herd

Date of bleeding	No. of samples	ELISA test P	S	N	Average P/N ratio ± S.E.	Specificity (%)
February 3, 1983	23	0	0	23	1.13 ± 0.040	100
June 13, 1983	77	0	0	77	1.04 ± 0.022	100
August 30, 1983	32	0	0	32	1.07 ± 0.028	100
October 18, 1983	10	0	0	10	1.06 ± 0.060	100
November 22, 1983	162	0	0	162	1.02 ± 0.013	100
Total	304	0	0	304	1.04 ± 0.010	100

P = positive
S = suspicious
N = negative

SPECIFICITY AND SENSITIVITY OF ELISA

All 304 sera collected from the IBR virus-free herd showed P/N ratios of less than 1.5 and revealed 100% specificity of the test (Table II). All of these sera were also negative with both the standard and modified SN tests. The sera of the 62 calves which were given an intranasal vaccine showed an ELISA test sensitivity of 27.4% and 100% at one month and six months postvaccination, respectively (Table III). All 303 samples which had a titer of $\geqslant$ 1:2 in the standard serum neutralization test gave P/N ratios in excess of 2.0 and a sensitivity of 100% (Table IV).

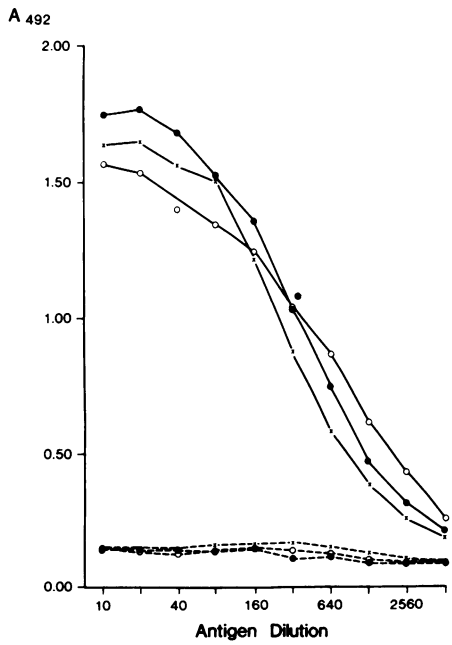

Fig. 2. IBRV-ELISA antigen titrations with negative (broken line) and positive (solid line) reference sera; o--o •--• Colorado strain of IBRV; x--x 108 strain of IBRV.

TABLE III. Comparison of ELISA with Standard SN Test on IBR Virus Intranasally Vaccinated Cattle

Sample	ELISA test				SN test		
	P[a]	S	N	% Sensitivity	P	N	% Sensitivity
Before vaccination (ten month old)[b]	1	0	61		0	62	
One month postvaccination	17	6	39	27.4	13	49	21.0
Six month postvaccination	62	0	0	100	59	3	95.2

[a]P = positive, S = suspicious, N = negative
[b]Majority of these animals, 35 of 37 tested, had maternal antibody against IBR virus at six weeks of age

TABLE IV. Relative Sensitivity of the ELISA Using Standard SN-Positive (≥1:2) Cattle Serum

Sample origin	ELISA			Sensitivity (%)
	P	S	N	
Export test	58	0	0	100
Quality control	74	0	0	100
Intranasal vaccinates	72	0	0	100
Infected herd	99	0	0	100
Total	303	0	0	100

P = positive
S = suspicious
N = negative

TABLE V. Test of Repeatability of Positive/Negative Ratio

Variable	Positive/Negative ratio[a]			Correlation coefficient[b]			
	Mean ± S.E.	Minimum	Maximum	Day 1	Day 2	Day 6	Day 9
Negative sera (N = 31)							
Day 1	1.034 ± 0.025	0.820	1.385				
Day 2	1.001 ± 0.028	0.710	1.350	0.8166			
Day 6	1.030 ± 0.024	0.785	1.350	0.8010	0.7060		
Day 9	0.970 ± 0.023	0.730	1.305	0.6602	0.6425	0.8103	
Day 12	0.938 ± 0.024	0.690	1.370	0.7948	0.7575	0.8885	0.8230
Positive sera (N = 38)							
Day 1	5.266 ± 0.453	2.115	11.710				
Day 2	5.312 ± 0.455	2.455	12.100	0.9916			
Day 6	4.623 ± 0.427	1.705	11.830	0.9726	0.9803		
Day 9	5.311 ± 0.862	1.810	12.665	0.9897	0.9865	0.9669	
Day 12	5.136 ± 0.468	1.830	12.190	0.9930	0.9915	0.9681	0.9925

[a]Analysis of variance of P/N ratios of repeated tests of both negative and positive sera are significant at the 1% level
[b]All correlation coefficients are significant at the 0.1% level

TABLE VI. Comparison of ELISA with Standard and Modified Serum Neutralization Tests

Source of Sample	ELISA			Standard SN		Modified SN		Total
	P	S	N	P	N	P	N	
Quality control	76	2	42	74	46	78	42	120
Export testing	109	22	91	58	164	109	113	222
Infected herd	100	2	19	99	22	104	17	121
Total	285	26	152	231	232	291	172	463

ELISA reaction	No.	Standard SN		Modified SN	
		P	N	P	N
P	285	231	54	274	11
S	26	0	26	14	12
N	152	0	152	3	149
Total	463	231	232	291	172

P = Positive
S = Suspicious
N = Negative

REPEATABILITY OF ELISA

Aliquots of 38 positive sera with various P/N ratios, including nine sera with borderline reactions, and 31 negative sera were tested at five different dates (Table V). All the negative sera showed negative ELISA results throughout the five repeat tests. All the positive sera showed positive reactions throughout the five repeat tests with the exception of one on day 6 and two on days 9 and 12 which showed suspicious reactions. These three sera had been selected from lower titer samples. Correlation coefficients between all combinations of repeated tests are also given in Table V. All correlation coefficients in Table V are significant at the 0.1% level. Positive sera gave superior correlations between the tests.

COMPARISON OF ELISA WITH STANDARD AND MODIFIED SN TESTS

Four hundred and sixty-three sera were used for comparison of the ELISA with the two SN tests (Table VI). The ELISA results showed 285 to be positive, 26 suspicious and 152 negative. The standard SN test detected 231 sera as positive and 232 sera as negative whereas the modified SN test showed 291 sera positive and 172 sera negative. All 231 sera which were standard SN positive were also positive both with the ELISA and the modified SN test. Of the 285 ELISA positive sera 274 were modified SN positive and 11 were negative, whereas of 291 sera which were positive with the latter test 274 were ELISA positive, 14 suspicious and three negative (Table VI). Figure 3 illustrates the P/N ratio frequency distribution of 304 negative sera which were assayed in Table I and 282 of 291 modified SN positive sera in Table VI (nine positive sera were duplicate samples in quality control test). Based on end point titrations, the modified SN test, on average was about 15 times more sensitive than the standard SN in detecting IBR viral neutralizing antibody.

DISCUSSION

The data presented indicate that the

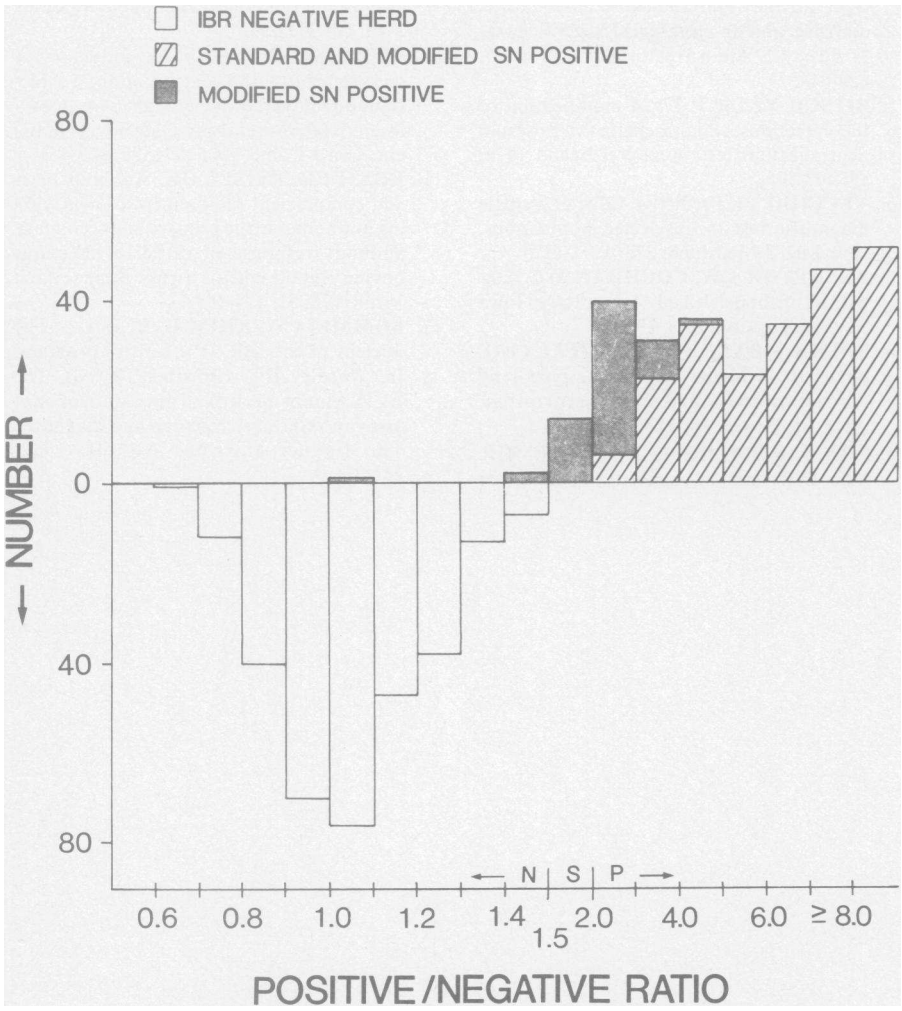

Fig. 3. Frequency distribution of P/N ratios of ELISA with sera collected from IBRV negative herd (n = 304) and modified SN test-positive sera (n = 282).

ELISA is a specific, sensitive and practical test for the detection of anti-IBR viral antibodies. This conclusion is in agreement with previous reports (1-3).

Many workers have determined the cut-off value of absorbance from known negative sera. As negative sera are usually selected by assays less sensitive than ELISA, sera with undetected antibody titers may be included in the reference negative serum pool thus increasing the possibility of false negative results (9). To avoid this limitation in the present study, the specificity of ELISA was determined by using a large number of sera (n = 304) collected from a known IBR virus-free herd. This should provide a more accurate indication of the diagnostic specificity of the test. Five of these sera were also used as reference negative pool in subsequent tests. Also, to reduce the inherent inter- and intra-group variations of A_{492} readings among plates and test dates, three replicate samples were tested on three separate plates at three different sample placement locations (Figure 1) and the P/N ratio was used as the cut-off value. All 304 sera collected from the IBR virus-free herd showed P/N ratios of less than 1.5 and all 303 samples which had a titer of $\geqslant$1:2 in the standard serum neutralization test gave P/N ratio of in excess of 2.0. Based on these data, a criterion for the interpretation of positive (P/N ratio $\geqslant$2.00) and negative (P/N ratio $<$1.50) was established. For those sera which were neither positive nor negative (P/N ratio $\geqslant$1.50 - $<$2.00), suspicious interpretations were given. These suspicious reactions are borderline and may represent very low concentrations of serum antibody produced in the early stage of IBR virus infection, which would subsequently become ELISA positive as shown in Table III. We have also observed suspicious reactions in sera used in a maternal antibody decay study, which subsequently become ELISA negative (Cho, Bradley and Yates, unpublished observations). In the present analysis of diagnostic sensitivity and specificity the suspicious results were considered as neither positive nor negative.

Based on the results obtained, the ELISA method appears to be superior to the standard SN test which is the official serological test for the detection of IBR viral antibody for regulatory purposes in Canada. Both tests have been shown to be specific, however, the ELISA method is more sensitive and practical. The present ELISA shows similar sensitivity to the modified SN test. In comparing the standard and modified SN tests, the latter proved to be on the average of about 15 times more sensitive than the former in detecting antibody titers and it allows the detection of complement-dependent IBR viral antibodies (5,10,11).

Due to its many advantages over the SN test, such as its sensitivity, its convenient technical features (1-3) plus its potential adaptability for automation, the ELISA method may be efficiently utilized as an alternative official test for the detection of IBR viral antibodies in cattle. In Switzerland, a micro-ELISA has been extensively utilized to detect IBR antibody (12).

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Fulltext

Dot immunogold filter kit for detecting ibr (infectious bovine rhinotracheitis) virus antibody and detection method thereof

List of publications Publication date

Application number 2012CN-0204875 Date 2012-06-20 Register

CN102692499 B Granted patent for invention 2015-01-28

AB

The invention discloses a dot immunogold filter kit for detecting an IBR (infectious bovine rhinotracheitis) virus antibody and a detection method thereof. The kit comprises a) an infectious bovine rhinotracheitis virus antigen, b) a gold marked goat anti-bovine antibody, c) a cleaning solution, and d) a confining liquid. The method comprises the following steps: dotting the infectious bovine rhinotracheitis virus antigen on a nitrocellulose film; closing, and adding a serum sample to be detected; cleaning, and detecting the infectious bovine rhinotracheitis virus antibody by using the gold marked goat anti-bovine antibody as colloidal gold marked protein. Detection of the infectious bovine rhinotracheitis virus antibody by adopting the kit disclosed by the invention has the advantages of specificity, sensitivity, quickness, reliability, intuitive effect, easily determined result and the like, special equipment is not required, and the detection result can be preserved for inspection.

EAB

The invention discloses a dot immunogold filter kit for detecting an IBR (infectious bovine rhinotracheitis) virus antibody and a detection method thereof. The kit comprises a) an infectious bovine rhinotracheitis virus antigen, b) a gold marked goat anti-bovine antibody, c) a cleaning solution, and d) a confining liquid. The method comprises the following steps: dotting the infectious bovine rhinotracheitis virus antigen on a nitrocellulose film; closing, and adding a serum sample to be detected; cleaning, and detecting the infectious bovine rhinotracheitis virus antibody by using the gold marked goat anti-bovine antibody as colloidal gold marked protein. Detection of the infectious bovine rhinotracheitis virus antibody by adopting the kit disclosed by the invention has the advantages of specificity, sensitivity, quickness, reliability, intuitive effect, easily determined result and the like, special equipment is not required, and the detection result can be preserved for inspection.

OAB

本发明公开一种检测IBR病毒抗体的金标免疫渗滤试剂盒及检测方法，具体包括以下步骤：所述试剂盒的组成包括：a）牛传染性鼻气管炎病毒抗原；b）金标记羊抗牛抗体；c）洗涤液；d）封闭液。先将牛传染性鼻气管炎病毒抗原点样于硝酸纤维素膜上，封闭后加待测血清样品，洗涤后用金标记羊抗牛抗体作为胶体金标记蛋白检测牛传染性鼻气管炎病毒抗体。采用本发明的试剂盒用于牛传染性鼻气管炎病毒抗体的检测,具有特异、敏感、快速可靠、效果直观、结果容易判断等优点，且不需要特殊仪器设备，检测结果可以保存备查。

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TECD Analysis of biological materials

PCLH

IC G01N-033/569* G01N-033/531

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Primary examiner: Li Hongyue

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CLMS

1. A gold-labeled immunodiafiltration kit for detecting infectious bovine rhinotracheitis virus antibodies, the kit comprising:

a. infectious bovine rhinotracheitis virus antigen;

b. gold-labeled goat anti-bovine antibodies;

c. washing liquid;

d. sealing liquid;

the preparation method of the infectious bovine rhinotracheitis virus antigen comprises the following steps: adopting a serum-free culture method to culture cattleCulturing infectious bovine rhinotracheitis virus on kidney passage cells, repeatedly freezing and thawing a culture solution of the infectious bovine rhinotracheitis virus for 3 times, centrifuging for 30min at 5000 r/min, taking supernatant, filling the supernatant into a dialysis bag, dialyzing in 0.01mol/L PBS (phosphate buffer solution) with pH of 7.4 until the culture solution is transparent and clear, and concentrating the dialysate; the serum-free culture method adopts a basic culture medium DMEM/F12=1:1 without adding serum, and the culture conditions are 37 ºC and 5% CO_2;

The confining liquid is 0.01mol/L PBS solution with pH7.2 containing 10g/L casein and 0.5mL/L Tween-20 or 0.01mol/L PBS solution with pH7.2 containing 5g/L casein and 0.5mL/L Tween-20; the washing solution is 0.01mol/L PBS buffer solution with pH7.2 containing 0.5ml/L Tween-20;

the method for detecting the infectious bovine rhinotracheitis virus antibody by using the gold-labeled immunodiafiltration kit comprises the following steps:

firstly, spotting the infectious bovine rhinotracheitis virus antigen on a nitrocellulose membrane, sealing with sealing liquid, adding a serum sample to be detected, washing with washing liquid, then adding a gold-labeled goat anti-bovine antibody, washing with the washing liquid, and observing the result, wherein if red spots appear at the spotting position, the spotting position is positive, and if the red spots do not appear, the spotting position is negative; preparing a nano colloidal gold solution from the gold-labeled goat anti-bovine antibody by adopting a trisodium citrate reduction method, and then adding the goat anti-bovine antibody into the nano colloidal gold solution; spotting the following on the nitrocellulose membrane in the kit at symmetrical positions: one point is 1 mu L of infectious bovine rhinotracheitis virus antigen as a detection point, and the other point is 1 mu L of staphylococcal protein A as a quality control point; drying at room temperature, adding 100 μ L of blocking solution, wherein the concentration of infectious bovine rhinotracheitis virus antigen is 0.95 mg/mL-1.81 mg/mL, and the concentration of staphylococcal protein A is 1 g/L; after the confining liquid is dried, 50-100 mu L of serum to be detected is added; adding a washing solution for washing after infiltration; adding 100 mu L of gold-labeled goat anti-bovine antibody; washing with washing solution after infiltration to remove unbound gold-labeled goat anti-bovine antibody; within 5-10 min, if red spots appear at the spotting position on the nitrocellulose membrane, the test is positive, if the red spots do not appear, the test is negative, the test is effective, the red spots appear at the quality control point, otherwise, the membrane is ineffective, and the membrane is spotted again.

OCLM

1.一种检测牛传染性鼻气管炎病毒抗体的金标免疫渗滤试剂盒，其特征在于，所述试剂盒的组成包括：

a. 牛传染性鼻气管炎病毒抗原；

b. 金标记羊抗牛抗体；
c. 洗涤液；
d. 封闭液；
所述牛传染性鼻气管炎病毒抗原的制备方法如下：采用无血清培养方法在牛肾传代细胞上培养牛传染性鼻气管炎病毒，将牛传染性鼻气管炎病毒的培养液反复冻融3次，5000 r/min离心30 min，取上清液装入透析袋中，置于0.01 mol/L pH7.4 PBS 缓冲液中透析，直至培养液透明清亮为止，然后将透析液浓缩；所述无血清培养方法采用不添加血清的基础培养基DMEM/F12=1:1，培养条件为37℃、5%CO_2；
所述封闭液为含10g/L酪蛋白、0.5mL/L吐温-20的0.01 mol/L pH7.2的PBS溶液或含5g/L酪蛋白、0.5mL/L 吐温-20的0.01 mol/L pH7.2的PBS溶液；所述洗涤液为含0.5ml/L 吐温-20的0.01mol/L pH7.2的PBS缓冲液；
利用所述金标免疫渗滤试剂盒检测牛传染性鼻气管炎病毒抗体的方法如下：
先将牛传染性鼻气管炎病毒抗原点样于硝酸纤维素膜上，用封闭液封闭后加待测血清样品，洗涤液洗涤，然后加金标记羊抗牛抗体，洗涤液洗涤，观察结果，若点样处出现红色斑点即为阳性，若不出现红色斑点即为阴性；所述金标记羊抗牛抗体采用柠檬酸三钠还原法制备纳米胶体金溶液，然后在纳米胶体金溶液中加入羊抗牛抗体；在试剂盒内硝酸纤维素膜上对称位置点样如下：一点为牛传染性鼻气管炎病毒抗原1μL作为检测点，另一点为葡萄球菌A蛋白1μL作为质控点；室温干燥后加入100μL封闭液，所述点样时牛传染性鼻气管炎病毒抗原浓度为0.95 mg/mL - 1.81mg/mL，葡萄球菌A蛋白浓度为1 g/ L；待封闭液干燥后，加入待检血清50μL - 100μL；渗入后加入洗涤液洗涤；加金标记羊抗牛抗体100μL；渗入后加入洗涤液洗涤，洗去未结合的金标记羊抗牛抗体；5 min-10min 内若在硝酸纤维素膜上点样处出现红色斑点即为阳性，若不出现红色斑点即为阴性，作为试验有效的标志，质控点应出现红色斑点，否则该膜无效，应重新点制膜。

DESC

TECHNICAL FIELD

[0002] The invention relates to a gold-labeled immunodiafiltration kit and a detection method for detecting an IBR virus antibody.

BACKGROUND ART

[0004] Infectious Bovine Rhinotracheitis (IBR) is a contagious disease of cattle caused by bovine herpes virus type i, and is characterized mainly by hyperpyrexia, dyspnea, inflammation of upper respiratory tract and tracheal mucosa, genital tract infection, conjunctivitis, and the like. Generally, the clinical morbidity of a cattle group is about 20% -30%, but the seropositive rate is much higher, and because the cattle with positive antibodies has the characteristics of latent infection and long-term detoxification of organisms, the cattle with positive antibodies are actually recessive carriers. IBRV has typical universal tropism, can invade various organs and tissues, causes various clinical symptoms, causes great economic loss for cattle raising industry, is one of important quarantine objects when cattle and cattle semen are imported in international animal trade and animal epidemic diseases which are highly concerned by import countries, and is classified as the second class of animal epidemic diseases by the Ministry of agriculture in China.

[0005] Infectious bovine rhinotracheitis almost spreads all over the world, and occurs in australia, new zealand and other countries of main dairy cow imports in China. The disease is reported for the first time in 1955 in fattening vegetable cattle in the state of colorado in the United states, IBRV is first separated from the imported dairy cattle in New Zealand in 1980 in China, and subsequent serological investigation proves that a certain proportion of IBR antibody positive cattle exist in cattle groups in a plurality of provinces and cities in China. In recent years, antibody-positive cows have been detected and the virus has been isolated to varying degrees from imported cows. Therefore, elimination of IBR antibody positive cattle and elimination of latently infected animals is the best and simplest method for controlling IBR, but the key problem is to establish a method for detecting IBR positive cattle first. At present, the national standards and industrial standards for detecting infectious bovine rhinotracheitis exist in China, and the national standards and the industrial standards comprise virus isolation through cell culture, PCR detection of virus nucleic acid, serum neutralization test of virus antibody and enzyme-linked immunosorbent assay (ELISA). The detection process is complicated, the virus detection needs 5d to 6d to report a positive result, and the fastest ELISA method in the serum antibody detection method also needs 2h to 4 h. And most of the reagents for detecting the IBR antibody are ELISA diagnostic kits imported from foreign countries, so that the price is high, the detection cost is increased, and meanwhile, special instruments such as an enzyme-labeling instrument and the like, the experimental skills of operators and certain experimental environmental conditions are required, so that the reagents are difficult to popularize in basic clinical practice.

DISCLOSURE

[0006] Disclosure of Invention

[0007] In order to solve the actual problems, the invention aims to provide a gold-labeled immunodiafiltration kit and a detection method for detecting an IBR virus antibody, and the kit has the advantages of specificity, sensitivity, rapidness, reliability, intuitive effect, easy judgment of results and the like, does not need special instruments and equipment, and can store and look up the detection results of a detection sample.

[0008] In order to achieve the purpose, the invention adopts the following technical scheme:

[0009] the invention firstly provides a gold-labeled immunodiafiltration kit for detecting IBR virus antibody, which comprises the following components:

[0010] a) infectious bovine rhinotracheitis virus antigen;

[0011] b) gold-labeled goat anti-bovine antibodies;

[0012] c) washing liquid;

[0013] d) and (4) sealing liquid.

[0014] The preparation method of the infectious bovine rhinotracheitis virus antigen comprises the following steps: culturing infectious bovine rhinotracheitis virus on bovine kidney passage cells by adopting a serum-free culture method, repeatedly freezing and thawing a culture solution of the infectious bovine rhinotracheitis virus for 3 times, centrifuging for 30min at 5000 r/min, taking supernatant, putting the supernatant into a dialysis bag, dialyzing in 0.01mol/L PBS buffer solution with pH of 7.4 until the culture solution is transparent and clear, and then concentrating the dialysate.

[0015] The serum-free culture method adopts a basal culture medium DMEM/F12 (1:1) without serum, and the culture conditions are 37 ℃ and 5% CO_2。

[0016] The blocking solution is a 0.01mol/L PBS solution with pH7.2 containing 10g/L casein and 0.5mL/L Tween-20 (Tween-20) or a 0.01mol/L PBS solution with pH7.2 containing 5g/L casein and 0.5mL/L Tween-20; the washing solution is 0.01mol/L PBS buffer solution with pH7.2 and containing 0.5ml/L Tween-20.

[0017] The invention also provides a method for detecting the infectious bovine rhinotracheitis virus antibody by using the gold-labeled immunodiafiltration kit, which comprises the steps of firstly spotting the infectious bovine rhinotracheitis virus antigen on a nitrocellulose membrane, adding a serum sample to be detected after sealing with a sealing liquid, washing with a washing liquid, then adding a gold-labeled goat anti-bovine antibody, washing with the washing liquid, and observing the result, wherein the result is positive if red spots appear at the spotting position, and the result is negative if the red spots do not appear.

[0018] Spotting the following on the nitrocellulose membrane in the kit at symmetrical positions: one point is 1 mu L of infectious bovine rhinotracheitis virus antigen as a detection point, and the other point is 1 mu L of staphylococcal protein A as a quality control point; drying at room temperature, and adding 100 μ L of sealing solution; after the confining liquid is dried, 50-100 mu L of serum to be detected is added; adding a washing solution for washing after infiltration; adding 100 mu L of gold-labeled goat anti-bovine antibody; washing with washing solution after infiltration to remove unbound gold-labeled goat anti-bovine antibody; within 5-10 min, if red spots appear at the spotting position on the nitrocellulose membrane, the test is positive, if the red spots do not appear, the test is negative, the test is effective, the red spots appear at the quality control point, otherwise, the membrane is ineffective, and the membrane is spotted again.

[0019] The concentration of the infectious bovine rhinotracheitis virus antigen during spotting is 0.95 mg/mL-1.81 mg/mL, and the concentration of staphylococcus A protein is 1 g/L.

[0020] The preparation method of the gold-labeled goat anti-bovine antibody comprises the following steps: preparing a nano colloidal gold solution by adopting a trisodium citrate reduction method, and then adding a goat anti-bovine antibody into the nano colloidal gold solution; the nano colloidal gold solution is prepared by adopting a conventional method: heating 100ml of 0.1ml/L chloroauric acid solution to boiling, rapidly adding 10g/L sodium citrate solution under the condition of magnetic heating and stirring, and carrying out constant-temperature reflux reaction under stirring to obtain the product. Taking nano colloidal gold sol, and using 0.1 mol/L K_2C0_3The pH was adjusted to 7.6. Then 2.5 mug goat anti-bovine antibody is added into each mL of colloidal gold solution, dropwise added under magnetic stirring, after 30min, 50g/L polyethylene glycol 20000 (PEG 20000) is added to the final concentration of 1g/L, stirring is continued for 15 min, and finally the mixture is placed in a refrigerator at 4 ℃ for overnight, and the solution is marked as the original volume. The next day, the solution was centrifuged at 5000 r/min for 10min, the supernatant was then centrifuged at 15000 r/min for 1h, and the supernatant was discarded. The pellet was returned to the original volume with resuspension buffer. Centrifuging at 15000 r/min for 30min, and discarding the supernatant. The precipitate was recovered to 1/40-1/5% of the original volume with colloidal gold diluent and stored in a refrigerator at 4 ℃.

[0021] Wherein the diameter of the prepared nano colloidal gold particles is 20nm-30 nm.

[0022] The adopted resuspension buffer is PBS buffer containing 10g/L Bovine Serum Albumin (BSA), 0.5g/L PEG 20000, 0.5mL/L Tween-20 and 0.01mol/L PH 7.2; the colloidal gold diluent used was PBS buffer containing 10g/L BSA, 0.5g/L PEG 20000, 50g/L sucrose, 0.5mL/L Tween-20, 0.01mol/L pH 7.2.

[0023] The kit is a plastic square small box, the volume of the box is 4.8cm multiplied by 3.0cm multiplied by 0.7cm, the box is divided into a bottom part and a cover part, and a circular hole with the diameter of 0.5cm is arranged on the cover surface. The box is internally provided with two layers, a nitrocellulose membrane (NC membrane) is covered towards the box, and a specially-made

absorbent paper pad is tightly attached under the NC membrane.

[0024] The invention has the beneficial effects that: the invention relates to a nano colloidal gold immune infiltration detection kit for detecting infectious bovine rhinotracheitis virus antibodies, which is researched and established by taking the infectious bovine rhinotracheitis virus cultured in a serum-free way as an antigen and adopting a nano colloidal gold immune labeling technology. The method is characterized in that a nitrocellulose membrane (NC membrane) is used as a carrier, an antigen is spotted on the NC membrane, a sample to be detected is added after the NC membrane is sealed by a sealing liquid, the sample is washed after being infiltrated, then the antibody marked by the nano colloidal gold is added, and the reaction signal of the antigen and the antibody is amplified by using the characteristic of developing color by the nano colloidal gold, so that the reaction result can be displayed on a solid phase carrier, namely the NC membrane. The method has the advantages of specificity, sensitivity, rapidness, reliability, intuitive effect, easy judgment of results and the like, does not need special instruments and equipment, and can store the detection results of the detection samples for retrospective examination. The nano colloidal gold immune infiltration detection method and the ELISA method are applied to simultaneously detect 300 clinical bovine serum samples of milk, and the detection result of the nano colloidal gold immune infiltration detection method is that the positive serum is 17 parts and the negative serum is 283 parts; the ELISA method showed that the positive serum was 18 parts and the negative serum was 182 parts. The positive rates of the nano colloidal gold immunogold filtration assay and the ELISA method are respectively 5.7% and 6.0%, and the detection coincidence rate of the nano colloidal gold immunofiltration assay and the ELISA method on a target positive sample reaches 94.4%.

[0025] The method established by the invention is used for detecting the infectious bovine rhinotracheitis virus antibody, has specificity, sensitivity, easy operation and convenient judgment of results, and has no cross reaction with positive serum of bluetongue, bovine akabane, bovine leukemia, bovine viral diarrhea mucosa disease, swine fever, porcine pseudorabies and porcine parvovirus disease. The successful establishment of the method can relatively quickly and simply judge whether the clinical cattle are only infected with IBR, and plays an important and positive role in effectively controlling the spread of the infectious bovine rhinotracheitis. At present, no report of detecting the infectious bovine rhinotracheitis by utilizing a nano colloidal gold immune labeling technology at home and abroad is seen, and the technology is expected to provide a quick, sensitive, simple, convenient and reliable method for detecting the infectious bovine rhinotracheitis of entry and exit (particularly entry cattle), so that the method is applied to entry cattle quarantine work, and simultaneously provides a basis for epidemiological investigation and clinical diagnosis of the disease in domestic cattle herds.

DRAWINGS DESCRIPTION

[0027] FIG. 1 is a cross-sectional test result chart of the optimal sample antigen concentration and the concentration of the nano-colloidal gold labeled goat anti-bovine antibody in the present invention; wherein: A-D-gold labeled goat anti-bovine IgG, which is 1/40, 1/20, 1/10 and 1/5 of the original volume respectively; 1-5-1 muL of coated IBR antigen, wherein the concentration is 0.1131mg/mL, 0.2263 mg/mL, 0.4525 mg/mL, 0.95 mg/mL and 1.81mg/mL respectively; and C, 1 mu L of quality control point-coating staphylococcus A protein, wherein the concentration is 1 g/L.

[0028] FIG. 2 is a graph showing the selection results of a blocking solution for antigen coating on an NC membrane according to the present invention; wherein: 1. blocking with 0.01mol/L PBS (pH7.2) containing 10g/L BSA and 5mL/L Tween-20; 2. blocking with 0.01mol/L PBS (pH7.2) containing 5g/L BSA and 5mL/L Tween-20; 3. blocking with 10g/L casein, 5mL/L Tween-20 and 0.01mol/L PBS (pH7.2); 4. blocking with 5g/L casein, 5mL/L Tween-20 and 0.01mol/L PBS (pH7.2); 5. blocking with 10g/L casein, 0.5mL/L Tween-20 and 0.01mol/L PBS of PH 7.2; 6. PBS containing 5g/L casein, 0.5mL/L Tween-20, 0.01mol/L pH7.2 blocks S-serum samples; c-quality control point.

INVENTION MODE

[0029] Detailed Description

[0030] The present invention will be described in further detail with reference to the attached drawings, but the present invention is not limited thereto.

[0031] 1. This example first prepares and concentrates infectious bovine rhinotracheitis virus antigen

[0032] On bovine kidney passaged cells (BK cells), DMEM/F12 (1:1) (purchased from Seimer Feishale Biochemical Co., Ltd.) as a basal medium without serum addition, 5% CO at 37 ºC was used$_2$ Culturing infectious bovine rhinotracheitis virus under the condition, repeatedly freezing and thawing the culture solution of IBR virus cultured in serum-free culture solution for 3 times, centrifuging at 5000 r/min for 30min, collecting supernatant, placing into dialysis bag, dialyzing in 0.01mol/L PBS buffer solution with pH of 7.4 overnight, and changingThe solution was repeated several times until the cell culture solution was clear. Then, the mixture was concentrated by centrifugation using a vacuum centrifugal concentrator. The optical density values at 260 nm and 280 nm were measured by UV spectrophotometry. And calculating the protein content of the virus protein liquid according to an empirical formula. Subpackaging and freezing at-20 ºC for later use.

[0033] 2. Preparation of goat anti-bovine antibody colloidal gold

[0034] 1) Preparing nano colloidal gold: preparing the nano colloidal gold sol by a citric acid reduction method. Adding 1mL of 10mL/L chloroauric acid (purchased from general chemical reagent factory of Shanghai city) into 100mL of deionized double distilled water to obtain a chloroauric acid solution with the concentration of 0.1mL/L, placing the chloroauric acid solution in a flask with a condensing device, heating to boil, rapidly adding 2 mL of 10g/L trisodium citrate (purchased from national pharmaceutical group chemical reagent Co., Ltd.) aqueous solution under magnetic heating and stirring, and continuing heating and boiling until the solution is wine-colored. The diameter of the obtained colloidal gold particles is (20-30) nm. After cooling, the mixture was stored in a brown bottle at 4 ºC in a refrigerator.

[0035] 2) Preparing a goat anti-bovine antibody nano colloidal gold marker: taking the nano colloidal gold solution with the required preparation amount and using 0.1 mol/L K_2C0_3(available from national pharmaceutical group chemical Co., Ltd.) to a pH of 7.6. Then 2.5. mu.g goat anti-bovine antibody (available from Santa Cruz Biotechnology, Germany) was added to each mL of the colloidal gold solution dropwise with magnetic stirring, 50g/L PEG 20000 was added to a final concentration of 1g/L after 30min, stirring was continued for another 15 min, and finally the mixture was left in a refrigerator at 4 ºC overnight. The next day, the solution was centrifuged at 5000 r/min for 10min, the supernatant was then centrifuged at 15000 r/min for 1h, and the supernatant was discarded. The pellet was returned to its original volume (volume after labeling with colloidal gold and before centrifugation) using resuspension buffer (PBS buffer containing 10g/L BSA, 0.5g/L PEG 20000, 0.5mL/L Tween-20, 0.01mol/L pH 7.2). Centrifuging at 15000 r/min for 30min, and discarding the supernatant. The precipitate was recovered to 1/40 in volume with a colloidal gold diluent (containing 10g/L BSA, 0.5g/L PEG 20000, 50g/L sucrose, 0.5mL/L Tween-20, 0.01mol/L PBS buffer pH 7.2), and stored in a refrigerator at 4 ºC.

[0036] 3. Manufacturing a reaction box: the reaction kit is a plastic square small box, the volume is 4.8cm multiplied by 3.0cm multiplied by 0.7cm, the reaction kit is divided into a bottom part and a cover part, and the cover surface is provided with a round hole with the diameter of 0.5 cm. Two layers were placed inside the box, the cover of which was a nitrocellulose membrane (NC membrane) (available from Schleicher & Schuell) against which was placed a specially made absorbent paper pad (available from Schleicher & Schuell).

[0037] 4. Using a reaction kit as a carrier, spotting the infectious bovine rhinotracheitis virus antigen prepared in the step a) on a nitrocellulose membrane, sealing, adding a serum sample to be detected, washing, and detecting the infectious bovine rhinotracheitis virus antibody by using the goat anti-bovine antibody nano colloidal gold prepared in the step b).

INVENTION MODE

[0038] Example 1

[0039] The symmetric positions on the NC membrane in the round well of the reaction cassette prepared in the above 3 are as follows: one point is that 1 muL of IBR antigen ((0.95-1.81) mg/mL) is used as a detection point, and the other point is that 1 muL of staphylococcal protein A (purchased from Shanghai biological products institute of Ministry of health) (1 g/L) is used as a quality control point; drying at room temperature, and adding 100 μ L of sealing solution; after the confining liquid is dried, adding 50-100 μ L of serum to be detected; adding 100 mu L of washing solution after infiltration, and repeating for 2-3 times; adding 100 mu L of goat anti-bovine antibody colloidal gold marker; and adding 100 mu L of washing solution for washing after infiltration, repeating for 2-3 times, and washing away unbound goat anti-bovine antibody colloidal gold markers. Within 5 min, the membrane is positive if red spots appear on the spot, and is negative if no red spots appear. As the mark for the test effectiveness, the quality control point should appear red spots, otherwise the test is ineffective, and the membrane should be spotted again.

INVENTION MODE

[0040] Example 2

[0041] The optimal antigen concentration and the optimal goat anti-bovine antibody colloidal gold marker concentration are selected, 1. mu.L of purified IBR antigen with 5 concentrations of 1.81mg/mL, 0.95 mg/mL, 0.4525 mg/mL, 0.2263 mg/mL and 0.1131mg/mL is spotted on the sample, 4 copies are repeated, 100. mu.L of clinically confirmed critical IBR positive serum by an ELISA method is added, and after washing, gold-labeled goat anti-bovine antibodies (1/40, 1/20, 1/10 and 1/5 in original volume) with different concentrations are respectively added for cross reaction. Screening the optimal antigen concentration and the optimal goat anti-bovine antibody colloidal gold marker concentration. As shown in fig. 1, the experiment performed in this example was: 1 mu L of IBR antigen is point-prepared and coated on round holes in the A-D reaction boxes in parallel and symmetrically, and the concentrations of 1-5 are 0.1131mg/mL, 0.2263 mg/mL, 0.4525 mg/mL, 0.95 mg/mL and 1.81mg/mL respectively; c in the round hole is a quality control point, 1 mu L of staphylococcus A protein is coated, and the concentration is 1 g/L; the A-D reaction boxes respectively represent that goat anti-bovine IgG colloidal gold markers with different concentrations are added on the round holes, and the A-D reaction boxes are 1/40, 1/20, 1/10 and 1/5 of the original volume in sequence. As can be seen from the results of fig. 1: the IBR antigen is coated by (0.95-1.81) mg/mL sample application, and when the using amount of the goat anti-bovine IgG colloidal gold marker is 1/10 of the original volume, the requirements of clear spot color and capability of detecting the minimum sample application antigen concentration of critical positive serum and the minimum gold marker goat anti-bovine antibody concentration are met. Therefore, 1/10 with a spotting antigen concentration of (0.95-1.81) mg/mL and a gold-labeled goat anti-bovine antibody concentration of the original volume was selected.

INVENTION MODE

[0042] Example 3

[0043] The formula of the sealing liquid is selected, as shown in fig. 2, and the experiment performed in this example is as follows: NC membranes prepared by IBR antigen spotting are respectively sealed by the following 6 sealing liquids: (1) 0.01mol/L PBS pH7.2 containing 10g/L BSA, 5mL/L Tween-20; (2) 0.01mol/L PBS pH7.2 containing 5g/L BSA, 5mL/L Tween-20; (3) PBS containing 10g/L casein, 5mL/L Tween-20 and 0.01mol/L pH7.2; (4) PBS (0.01 mol/L pH7.2) containing 5g/L casein and 5mL/L Tween-20; (5) PBS containing 10g/L casein, 0.5mL/L Tween-20 and 0.01mol/L PH 7.2; (6) 0.01mol/L PBS pH7.2 containing 5g/L casein, 0.5mL/L Tween-20. Through repeated experimental tests, the results are as follows: the 5 th or 6 th blocking solution is adopted, namely PBS containing 10g/L casein, 0.5mL/L Tween-20 and 0.01mol/L PH7.2 or PBS containing 5g/L casein, 0.5mL/L Tween-20 and 0.01mol/L PH7.2 is used as the blocking solution, the blocking effect is best, and the selection requirement of the optimal blocking solution with clear spot color development and lighter background is met.

[0044] It will be understood that numerous changes in detail may be effected therein without departing from the scope and spirit of the invention, and it is intended that all such changes be effected by one skilled in the art without departing from the scope and spirit of the invention.

ODES

[0001] 技术领域

[0002] 本发明涉及一种检测IBR病毒抗体的金标免疫渗滤试剂盒及检测方法。

[0003] 背景技术

[0004] 牛传染性鼻气管炎（Infectious bovine rhinotracheitis，IBR），是由牛疱疹病毒I型引起的牛的一种接触性传染病，以高热、呼吸困难、上呼吸道及气管粘膜发炎以及生殖道感染、结膜炎等为主要特征。一般牛群临床发病率约20%-30%，但血清反应阳性率要高得多，由于本病有潜伏感染和机体长期排毒的性质，抗体阳性牛实际上就是隐性带毒者。IBRV具有典型的泛嗜性，能侵袭多种器官和组织，引起多种临床症状，给养牛业造成较大的经济损失，是国际动物贸易中进口种牛、牛精液时的重点检疫对象和进口国应高度关注的动物疫病之一，我国农业部将其列为二类动物疫病。

[0005] 牛传染性鼻气管炎几乎遍布世界各地，我国主要奶牛进口国澳大利亚、新西兰等国均有发生。该病于1955年在美国科罗拉多州的育肥菜牛被首次报道，我国于1980年从新西兰进口的奶牛体内首次分离到IBRV，随后的血清学调查证实我国多个省市的牛群中均有一定比例的IBR抗体阳性牛存在。近几年从国外进口奶牛中也不同程度的检出抗体阳性牛和分离出该病毒。因此清除IBR抗体阳性牛，排除潜伏感染动物是控制IBR最好最简单的方法，但关键问题是要率先建立一种检测IBR阳性牛的方法。目前，国内已有的针对牛传染性鼻气管炎检测的国家标准和行业标准，包括经细胞培养分离病毒，病毒核酸的PCR检测、病毒抗体的血清中和试验和酶联免疫吸附试验（ELISA）。检验过程繁琐，病毒检测需要5d-6d才能报告阳性结果，血清抗体检测方法中最快速的ELISA方法也需要2h-4h。而且现行检测IBR抗体的试剂多为从国外进口的ELISA诊断试剂盒，价格昂贵推高了检测成本，同时还需要酶标仪等特定仪器、操作者的实验技能和一定的试验环境条件，难以在基层临床中推广。

[0006] 发明内容

[0007] 为了解决上述实际问题，本发明的目的在于提供一种检测IBR病毒抗体的金标免疫渗滤试剂盒及检测方法，本发明具有特异、敏感、快速可靠、效果直观、结果容易判断等优点，且不需要特殊仪器设备，检测样品的检测结果可以保存备查。

[0008] 为达到上述目的，本发明采用以下技术方案：

[0009] 本发明首先提供了一种检测IBR病毒抗体的金标免疫渗滤试剂盒，所述试剂盒的组成包括：

[0010] a）牛传染性鼻气管炎病毒抗原；

[0011] b）金标记羊抗牛抗体；

[0012] c）洗涤液；

[0013] d）封闭液。

[0014] 所述牛传染性鼻气管炎病毒抗原的制备方法如下：采用无血清培养方法在牛肾传代细胞上培养牛传染性鼻气管炎病毒，将牛传染性鼻气管炎病毒的培养液反复冻融3次，5000 r/min离心30 min，取上清液装入透析袋中，置于0.01 mol/L pH7.4 PBS 缓冲液中透析，直至培养液透明清亮为止，然后将透析液浓缩。

[0015] 所述无血清培养方法采用不添加血清的基础培养基DMEM/F12 （1:1），培养条件为37℃、5%CO_2。

[0016] 所述封闭液为含10g/L酪蛋白、0.5mL/L 吐温-20（Tween-20）的0.01 mol/L pH7.2的PBS溶液或含5g/L酪蛋白、0.5mL/L Tween-20的0.01 mol/L pH7.2的PBS溶液；所述洗涤液为含0.5ml/L Tween-20的0.01mol/L pH7.2的PBS缓冲液。

[0017] 本发明还提供了一种利用所述金标免疫渗滤试剂盒检测牛传染性鼻气管炎病毒抗体的方法，先将牛传染性鼻气管炎病毒抗原点样于硝酸纤维素膜上，用封闭液封闭后加待测血清样品，洗涤液洗涤，然后加金标记羊抗牛抗体，洗涤液洗涤，观察结果，若点样处出现红色斑点即为阳性，若不出现红色斑点即为阴性。

[0018] 在试剂盒内硝酸纤维素膜上对称位置点样如下：一点为牛传染性鼻气管炎病毒抗原1μL作为检测点，另一点为葡萄球菌A蛋白1μL作为质控点；室温干燥后加入100μL封闭液；待封闭液干燥后，加入待检血清50-100μL；渗入后加入洗涤液洗涤；加金标记羊抗牛抗体100μL；渗入后加入洗涤液洗涤，洗去未结合的金标记羊抗牛抗体；5 min-10min 内若在硝酸纤维素膜上点样处出现红色斑点即为阳性，若不出现红色斑点即为阴性，作为试验有效的标志，质控点应出现红色斑点，否则该膜无效，应重新点制膜。

[0019] 所述点样时牛传染性鼻气管炎病毒抗原浓度为0.95 mg/mL - 1.81mg/mL，葡萄球菌A蛋白浓度为1 g/ L。

[0020] 所述的金标记羊抗牛抗体，其制备方法如下：采用柠檬酸三钠还原法制备纳米胶体金溶液，然后在纳米胶体金溶液中加入羊抗牛抗体；纳米胶体金溶液采用常规制备：将100ml 0.1ml/L氯金酸溶液加热至沸腾，在磁力加热搅拌条件下迅速加入10g/L柠檬酸钠溶液，在搅拌下恒温回流反应制得。取纳米胶体金溶胶，用0.1 mol/L K_2C0_3调pH值至7.6。然后在每mL胶体金溶液中加入2.5μg羊抗牛抗体，磁力搅拌状态下逐滴加入，30 min后加入50g/L 聚乙二醇 20 000（PEG 20 000)至终浓度1g/L，再继续搅拌15 min，最后置4℃冰箱过夜，以此液标记为原体积。次日将此液以5 000 r/min离心10min，取上清液15 000 r/min离心1h，弃上清。在沉淀用重悬缓冲液恢复至原体积。15 000 r/min离心30min，弃上清。沉淀用胶体金稀释液恢复至原体积的1/40-1/5，置4℃冰箱保存。

[0021] 其中制备的纳米胶体金颗粒的直径为20nm-30nm。

[0022] 所采用的重悬缓冲液为含10g/L 牛血清白蛋白（BSA）、0.5g/L PEG 20 000、0.5mL/L Tween-20、0.01mol/L PH7.2的PBS缓冲液；所采用的胶体金稀释液为含10g/L BSA、0.5g/L PEG 20 000、50g/L蔗糖、0.5mL/L Tween-20、0.01mol/L PH7.2的PBS缓冲液。

[0023] 所述试剂盒为塑料正方形小盒，体积为4.8cm×3.0cm×0.7cm，分底和盖两部分，盖面有直径0.5cm 圆孔。盒内有两层，朝盖的为硝酸纤维素膜（NC膜），NC膜下紧贴特制的吸水纸垫。

[0024] 本发明的有益效果是：本发明是以无血清培养的牛传染性鼻气管炎病毒作为抗原，采用纳米胶体金免疫标记技术，研究建立检测牛传染性鼻气管炎病毒抗体的纳米胶体金免疫渗滤检测试剂盒。采用硝酸纤维素膜（NC膜）为载体，先将抗原点制于NC膜上，经封闭液封闭后加入待测样品，待样品渗入后洗涤，再加入纳米胶体金标记的抗体，应用纳米胶体金显色的特点将抗原抗体反应信号放大，使反应结果能够在固相载体——NC膜上得以显示。该方法具有特异、敏感、快速可靠、效果直观、结果容易判断等优点，且不需要特殊仪器设备，检测样品的检测结果可以保存用于回顾性检查。通过应用本发明纳米胶体金免疫渗滤检测法和ELISA方法同时对300份临床奶牛血清样品进行检测，用纳米胶体金免疫渗滤检测法检测结果阳性血清为17份，阴性血清为283份；ELISA法的检测结果阳性血清为18份，阴性血清为182份。纳米胶体金免疫渗滤检测法和ELISA方法的阳性率分别为5.7%和6.0%，两者对目标阳性样品检测符合率达94.4%。

[0025] 本发明建立的方法用于牛传染性鼻气管炎病毒抗体检测，具有特异、敏感、易于操作、结果便于判断，而且与蓝舌病、牛赤羽病、牛白血病、牛病毒性腹泻粘膜病、猪瘟病、猪伪狂犬病和猪细小病毒病阳性血清均无交叉反应。该方法的成功建立，能够相对快速简单地对临床牛只是否感染IBR的情况做出判断，将为有效控制牛传染性鼻气管炎的传播发挥重要而积极的作用。目前未见国内外利用纳米胶体金免疫标记技术检测牛传染性鼻气管炎的报道，该技术有望为出入境（特别是入境种牛）的牛传染性鼻气管炎检测提供一种快速灵敏、简便可靠的方法，从而在进境种牛隔离检疫工作中得到应用，同时也为国内牛群中该病的流行病学调查、临床诊断提供依据。

[0026] 附图说明

[0027] 图1为本发明最佳点样抗原浓度及纳米胶体金标记羊抗牛抗体浓度的交叉试验结果图；其中：A～D-金标羊抗牛IgG，分别为原体积的1/40，1/20，1/10，1/5.；1～5 -包被IBR抗原1μL，浓度分别为0.1131mg/mL，0.2263 mg/mL，0.4525 mg/mL，0.95 mg/mL，1.81 mg/mL；C，质控点-包被葡萄球菌A蛋白 1μL，浓度为 1 g/L。

[0028] 图2 为本发明NC膜上用于抗原包被的封闭液的选择结果图；其中：1.含10g/L BSA、5mL/L Tween-20的0.01 mol/L pH7.2的PBS封闭；2.含5g/L BSA、5mL/L Tween-20的0.01mol/L pH7.2的PBS封闭；3.含10g/L酪蛋白、5mL/L Tween-20的0.01 mol/L pH7.2的PBS封闭；4.含5g/L酪蛋白、5mL/L Tween-20的0.01 mol/L pH7.2的PBS封闭；5.含10g/L酪蛋白、0.5mL/L Tween-20的0.01 mol/L PH7.2的PBS封闭；6.含5g/L酪蛋白、0.5mL/L Tween-20的0.01 mol/L pH7.2的PBS封闭. S - 血清样品； C - 质控点。

[0029] 具体实施方式

[0030] 下面结合附图对本发明作进一步详细的说明，但不应理解为是对本发明进行的限定。

[0031] 1、本实施例首先制备和浓缩牛传染性鼻气管炎病毒抗原

[0032] 在牛肾传代细胞（BK细胞）上，采用不添加血清的基础培养基DMEM/F12 (1:1)（购自赛默飞世尔生物化学制品（北京）有限公司），37℃、5%CO_2 条件下培养牛传染性鼻气管炎病毒，将无血清培养液培养的IBR病毒培养液反复冻融3次，5 000 r/min离心30 min，取上清液装入透析袋中，置于0.01 mol / L pH7.4 PBS 缓冲液中透析过夜，其间换液数次，直到细胞培养液透明清亮为止。然后用真空离心浓缩仪离心浓缩。用紫外分光光度计测其在260 nm 和280 nm 处光密度值。根据经验公式计算病毒蛋白液的蛋白含量。分装后于-20℃冻存备用。

[0033] 2、羊抗牛抗体胶体金的制备

[0034] 1）纳米胶体金的制备：采用柠檬酸还原法制备纳米胶体金溶胶。取10mL/L氯金酸（购自上海市试剂总厂化学试剂一厂）1 mL加入到100 mL去离子双蒸水中，得到的氯金酸溶液的浓度为0.1mL/L，置于带冷凝装置的烧瓶中加热至沸腾，磁力加热搅拌下快速加入10g/L 柠檬酸三钠（购自国药集团化学试剂有限公司）水溶液2 mL，继续加热煮沸直到溶液呈葡萄酒色为止。得到的胶体金颗粒直径为（20-30）nm。 冷却后置棕色瓶于4℃冰箱保存。

[0035] 2）羊抗牛抗体纳米胶体金标记物的制备：取需制备量的纳米胶体金溶液，用0.1 mol/L K_2C0_3（购自国药集团化学试剂有限公司）调pH值至7.6。然后在每mL胶体金溶液加入2.5μg羊抗牛抗体（购于德国Santa Cruz Biotechnology公司），磁力搅拌状态下逐滴加入，30 min后加入50g/L PEG 20 000至终浓度1g/L，再继续搅拌15 min，最后置4℃冰箱过夜。第二天将此液以5 000 r/min离心10min，取上清液15 000 r/min离心1h，弃上清。沉淀用重悬缓冲液（含10g/L BSA、0.5g/L PEG 20 000、0.5mL/L Tween-20、0.01mol/L PH7.2的PBS缓冲液）恢复至原体积（指胶体金标记后、离心前的体积）。15 000 r/min离心30min，弃上清。沉淀用胶体金稀释液（含10g/L BSA、0.5g/L PEG20 000、50g/L蔗糖、0.5mL/L Tween-20、0.01mol/L PH7.2的PBS缓冲液）恢复至原体积的1/40，置4℃冰箱保存。

[0036] 3、反应盒的制作：反应试剂盒为塑料正方形小盒，体积为4.8cm×3.0cm×0.7cm，分底和盖两部分，盖面有直径0.5cm 圆孔。盒内有两层，朝盖的为硝酸纤维素膜（NC膜）（购于Schleicher & Schuell公司），NC膜下紧贴特制的吸水纸垫(购于Schleicher & Schuell公司)。

[0037] 4、应用反应试剂盒作为载体，先将步骤a)中制备的牛传染性鼻气管炎病毒抗原点样于硝酸纤维素膜上，封闭后加待测血清样品，洗涤后用步骤b)中制备的羊抗牛抗体纳米胶体金检测牛传染性鼻气管炎病毒抗体。

[0038] 实施例1

[0039] 在上述3中制备的反应盒圆孔内NC膜上对称位置点如下两点：一点是IBR抗原(（0.95～1.81）mg/mL) 1μL作为检测点，另一点是葡萄球菌A蛋白（购于卫生部上海生物制品研究所）（1g/L）1μL作为质控点；室温干燥后加入100μL封闭液；待封闭液干燥后，加入待检血清（50-100）μL；渗入后加入洗涤液100μL洗涤，重复2-3遍；加羊抗牛抗体胶体金标记物100μL；渗入后加入洗涤液100μL洗涤，重复2-3遍，洗去未结合的羊抗牛抗体胶体金标记物。5 min内若在膜上点样处出现红色斑点即为阳性，若不出现红色斑点即为阴性。作为试验有效的标志，质控点应出现红色斑点，否则该试验无效，应重新点制膜。

[0040] 实施例2

[0041] 最佳抗原浓度和最佳羊抗牛抗体胶体金标记物浓度的选择，分别以1.81 mg/mL、0.95 mg/mL、0.4525 mg/mL、0.2263 mg/mL和0.1131mg/mL 5种浓度的纯化IBR抗原1μL点样，重复4份，加入临床上经ELISA方法确认的临界IBR阳性血清100μL，洗涤后分别加入不同浓度的金标羊抗牛抗体（分别为原体积的1/40，1/20，1/10，1/5），进行交叉反应。筛选最佳抗原浓度和最佳羊抗牛抗体胶体金标记物浓度。如图1所示，本实施例所做的实验为：A～D反应盒内圆孔上，平行对称点制和包被IBR抗原1μL，1～5浓度分别为0.1131mg/mL，0.2263 mg/mL，0.4525 mg/mL，0.95 mg/mL，1.81 mg/mL；圆孔内C为质控点，包被葡萄球菌A蛋白 1μL，浓度为 1 g/L；A～D反应盒分别代表圆孔上加入不同浓度的羊抗牛IgG胶体金标记物，A～D依次为原体积的1/40，1/20，1/10，1/5。由图1结果显示可见：IBR抗原以（0.95～1.81）mg/mL 点样包被，羊抗牛IgG胶体金标记物使用量为原体积1/10时，符合斑点颜色清晰而且是能检出临界阳性血清的最低点样抗原浓度和最低金标羊抗牛抗体浓度的要求。因此，选择点样抗原浓度为（0.95 - 1.81） mg/mL，金标羊抗牛抗体浓度为原体积的1/10。

[0042] 实施例3

[0043] 封闭液的配方选择，如图2所示，本实施例所做的试验为：经IBR抗原点样制备的NC膜分别用以下6种封闭液进行封闭：（1）含10g/L BSA、5mL/L Tween-20的0.01 mol/L pH7.2的PBS；（2）含5g/L BSA、5mL/L Tween-20的0.01mol/L pH7.2的PBS；（3）含10g/L酪蛋白、5mL/L Tween-20的0.01 mol/L pH7.2的PBS；（4）含5g/L酪蛋白、5mL/L Tween-20的0.01 mol/L pH7.2的PBS；（5）含10g/L酪蛋白、0.5mL/L Tween-20的0.01 mol/L PH7.2的PBS；（6）含5g/L酪蛋白、0.5mL/L Tween-20的0.01 mol/L pH7.2的PBS。经过反复实验测试，结果为：采用第5种或第6种封闭液，即以含10g/L酪蛋白、0.5mL/L Tween-20的0.01 mol/L PH7.2的PBS或含5g/L酪蛋白、0.5mL/L Tween-20的0.01 mol/L pH7.2的PBS作为封闭液，封闭效果最好，符合斑点显色清楚且背景较浅的最适封闭液选择要求。

[0044] 可以理解，很多细节的变化是可能的，但这并不因此违背本发明的范围和精神，任何所属技术领域的普通技术人员对其所做的适当变化，皆应视为不脱离本发明专利的范畴。