UNIVERSIDADE FEDERAL DA BAHIA
INSTITUTO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM IMUNOLOGIA

DISSERTAÇÃO DE MESTRADO

ESTUDOS DE MOLÉCULAS EFETORAS DA RESPOSTA IMUNE CELULAR E HUMORAL EM PEQUENOS RUMINANTES CONTRA *Corynebacterium pseudotuberculosis* COMO MARCADORES EM ENSAIOS DE IMUNODIAGNÓSTICO

MIRIAM FLORES REBOUÇAS

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ORIENTADOR: DR. ROBERTO MEYER
CO-ORIENTADOR: DR. RICARDO PORTELA

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Dissertação apresentada ao Programa de Pós-Graduação em Imunologia, Instituto de Ciências da Saúde, Universidade Federal da Bahia, como requisito parcial para a obtenção do grau de Mestre em Imunologia.

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MIRIAM FLORES REBOUÇAS

Estudos de moléculas efetoras da resposta imune celular e humoral em pequenos ruminantes contra *Corynebacterium pseudotuberculosis* como marcadores em ensaios de imunodiagnóstico.

Dissertação apresentada como requisito parcial para a obtenção do Título de Mestre em Imunologia, Universidade Federal da Bahia, para a seguinte banca examinadora:

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ñTudo o que um sonho precisa para ser realizado é alguém que acredite que ele possa ser realizado.ò

Roberto Schinyashiki.

DE ABREVIATURAS E SIGLAS

BHI	Brain heart infusion.
PLD	Fosfolipase D.
LC ou CLA	Linfadenite caseosa.
E	Especificidade.
ELISA	Ensaio imunoenzimático (Enzyme Linked Immunosorbent Assay).
FP	Número de amostras falso positivas.
FN	Número de amostras falso negativas.
H	Hora.
IL	Interleucina .
IFN-gamma	Interferon gama.
IgG	Imunoglobulina G.
OD	Densidade óptica.
PBS	Tampão fosfato salino.
PBST	Tampão fosfato salino + Tween 20.
S	Sensibilidade.
SDS	Duodecil sulfato de sódio.
SDS-PAGE	Gel de poliacrilamida com duodecil sulfato de sódio.
Th1	Células T helper 1.
Th2	Células T helper 2.
TMB	Tetramehtylbenzidine.
TN	Número de amostras verdadeiramente negativas (truly negatives).
TNFα	Fator de necrose tumoral alfa (Tumor Necrosis factor).
TP	Número de amostras verdadeiramente positivas (truly positives).
TPP	Tree-Phase Partitioning.
PCR	Reação de cadeia em polimerase.

SUMÁRIO

1.1. Prevalência da doença

A Linfadenite caseosa (LC) ocorre em todas as áreas onde há significativa população de ovinos e caprinos no mundo, entretanto existem inúmeras dificuldades na obtenção de dados para se definir a prevalência desta doença, pricipalmente devido à sua característica essencialmente crônica e à sintomatologia não específica, escassa ou até mesmo ausente. Além disso, outros agentes etiológicos que levam à formação de granulomas semelhantes, tais como *Arcanobacterium pyogenes*, *Streptococcus spp* e *Staphylococcus spp*. Adicionalmente, na maioria dos inquéritos epidemiológicos, os dados baseiam-se em animais provenientes de abatedouros, que não são remetidos para o abate quando apresentam a forma clínica da doença (Unanian *et. al.* , 1985).

No Brasil, a doença é altamente freqüente no Nordeste. Estima-se que a maioria dos rebanhos está infectada e que a prevalência clínica atinge até 30% dos animais (Costa Filho *et. al.* , 1974). Unanian e colaboradores (1985), através de levantamento realizado em rebanho caprino no Ceará, relataram que a LC é responsável por 27,7% dos granulomas encontrados em 41,65% dos animais. Um dos registros de prevalência feitos na Bahia foi conduzido por Meyer (2004), no semi-árido baiano, obtendo um percentual médio de 46,66%.

1.2. Agente etiológico

C. pseudotuberculosis é uma bactéria caracterizada como um bacilo Gram positivo, podendo apresentar uma forma cocóide. Este microorganismo é anaeróbio facultativo, imóvel, não esporulado e fermentativo. É uma bactéria mesofílica, cuja temperatura ideal de crescimento é 37°C por 48 horas em uma atmosfera de 5% de gás carbônico, em meios de cultivo enriquecido com sangue, soro animal ou proteínas vegetais (Benham *et. al.*, 1962; Cameron & Swart, 1965; Selim, 2001). No ágar sangue, formam-se colônias pequenas de coloração branca acinzentada, opacas e friáveis. Após vários dias de incubação, as colônias podem alcançar 3mm de diâmetro e são de coloração creme, produzindo ƀ hemólise característica após um período de 48 a 72h de incubação (Quinn *et. al*., 1994).

s é capaz de permanecer viável no ambiente por longos períodos. O microorganismo pode alojar-se em superfícies e continuamente infectar animais por contato direto. Portanto, a alta persistência do mesmo no ambiente parece ser o principal fator responsável pela sua manutenção dentro de um rebanho (Ellis *et. al.*, 1987).

1.3. Achados clínicos da linfadenite caseosa em pequenos ruminantes

Por ser uma doença caracteristicamente crônica, a LC possui em geral um longo período de incubação em pequenos ruminantes, se iniciando com um abscesso localizado e indolor, que aumenta lentamente de volume em linfonodos internos ou em vísceras. Na maioria das vezes, nenhum sinal da doença torna-se perceptível na fase inicial da doença, dificultando ou impossibilitando o diagnóstico clínico precoce da enfermidade (Batey, 1986). Desta forma, uma infecção inicial pode não originar nenhum sinal clínico ou pode ser acompanhada por sintomas não patognomônicos, tais como febre, anorexia e anemia. Os animais com abscessos superficiais não exibem nenhum efeito patológico óbvio, a menos que a localização do abscesso interfira em funções com deglutição ou respiração, podendo uma abscedação reincidivar no mesmo local. Os abscessos pulmonares, hepáticos e renais causam comumente a ñsíndrome da ovelha magraò, provocando rapidamente uma progressiva debilitação e perda de peso. A incidência aumenta constantemente com a idade, a doença clínica é mais prevalente nos adultos e até 40% dos animais em um rebanho pode apresentar esses abscessos superficiais (Manual Merck, 2001).

A lesão macroscópica típica é um abscesso discreto, distendido por exsudato purulento amarelo esverdeado ou branco espesso e frequentemente seco. Embora seja uma infecção principalmente dos ovinos e caprinos, a doença ocorre esporadicamente também nos equinos, bovinos, búfalos, ruminantes silvestres, primatas, suínos, aves domésticas, e raramente no homem (Yeurhuam *et. al*., 1997).

1.4. Moléculas importantes na interação com os hospedeiros

A patogenia dessa bactéria ainda não está completamente esclarecida, mas dois fatores de virulência já foram descritos; os lipídios de parede e a exotoxina fosfolipase D (PLD), importantes na imunopatogenia da doença (Brown & Olander, 1997).

O ácido micólico, presente em *Mycobacterium tuberculosis,* se assemelha muito aos lipídios integrados à parede celular de *C. pseudotuberculosis*. A presença desses lipídios é associada ao seu crescimento característico em meio líquido, sob a forma de flocos característicos (Jolly, 1966). Essa camada dificulta a fagocitose da bactéria, aumentando sua virulência, e também se relaciona à citotoxicidade (Batey, 1986; Songer *et. al*., 1990).

As ações deletérias destes lipídios podem ocorrer de duas formas distintas: ação tóxica direta sobre as células fagocitárias, o que consiste um papel importante na patogenicidade da doença, e resistência à ação bactericida de enzimas hidrolíticas presentes nos lisossomas, permitindo a sobrevivência da bactéria dentro da célula (Muckle & Gyles, 1983; Willianson, 2001).

Os lipídios da parede bacteriana parecem contribuir, além disso, para a formação do abscesso. Infecções artificiais em camundongos demonstraram uma relação direta entre o conteúdo lipídico das linhagens de *C. pseudotuberculosis* testadas e a capacidade de geração de uma infecção crônica (Muckle & Gyles, 1983).

A PLD vem sendo identificada como uma potente exotoxina de *C. pseudotuberculosis* e um fator chave para o desenvolvimento da LC. Diversos estudos usando a exotoxina bruta ou parcialmente purificada demonstraram que a atividade da PLD sobre a esfingomielina, que constitui a membrana das células do endotélio muscular, parece ser importante no processo de disseminação dessa bactéria da área infectada para os linfonodos. Bactérias sem a expressão desse gene não são capazes de disseminar a doença no organismo (McNamara *et. al*., 1994). Similarmente, a presença de anticorpos específicos para PLD limitam o progresso da doença clínica (Alves & Olander, 1999; Baird *et. al*., 2007).

O gene *pld* de *C. pseudotuberculosis* já foi clonado e seqüenciado (McNamara *et. al*., 1994). Estudos relatam que se trata de uma proteína de 31,5 KDa, que apresenta diversas atividades biológicas, incluindo dermonecrose (Muckle & Gyles, 1983), lise de eritrócitos na presença de um fator extracelular de

961) e inibição da hemólise pela toxina beta estafilocócica. Essas duas últimas propriedades são utilizadas em testes laboratoriais para identificação de *C. pseudotuberculosis* no teste da inibição da ação anti-hemolisina e teste da inibição da hemólise sinérgica (Zaki, 1976).

Além da PLD, outros antígenos capazes de induzir a resposta imune têm sido relatados, dentre ele antígenos que são secretados pelo microorganismo e encontrados no sobrenadante da cultura, à semelhança da PLD, e antígenos associados à célula bacteriana, podendo estes ser obidos por diversos métodos como a sonicação, extração com detergentes e extração com o uso do cloreto de sódio (Moura-Costa, 2002).

1.5. Resposta imunológica a *Corynebacterium pseudotuberculosis*

A imunidade a *C. pseudotuberculosis* é atribuída a mecanismos humorais e celulares (Lan *et. al*., 1998). A proteção por anticorpos já foi demonstrada por Cameron & Engelbrecht (1971) através de imunização passiva em camundongos com soro de coelho imunizados contra a bactéria. O efeito protetor foi associado à capacidade dos anticorpos atuarem contra a disseminação da bactéria, neutralizando exotoxinas.

C. pseudotuberculosis é um patógeno intracelular facultativo que se multiplica dentro de macrófagos. Em estudo para avaliar a interação de macrófagos caprinos com este microorganismo, foi observado que apesar de ocorrer à fusão do fagossoma com o lisossoma, a bactéria não era destruída. Esse patógeno aparentemente resiste à morte e digestão no interior dos fagócitos devido à ação de seus lipídeos de parede (Tashjiam & Campbell, 1983).

Os granulomas, principal manifestação clínica da doença, são formados por várias camadas. Internamente é encontrado um centro necrótico, seguido por uma camada rica em macrófagos, uma zona com predominância de linfócitos T $CD4^+$, $CD8^+$ e Tyŭ e uma zona rica em linfócitos B, limitada externamente por uma cápsula fibrótica. Nas lesões imaturas existe uma predominância de células T $CD4^+$ e, nas lesões maduras, T $CD8^+$ e Tyŭ (Pépin *et. al*., 1994).

A resistência contra bactérias intracelulares facultativas está relacionada à atividade de células T $CD4^+$ e, mais especificamente, à subpopulação Th1, pelo aumento da atividade microbicida dos macrófagos induzida pela produção de INF-y

CD8$^+$ na resistência a este tipo de infecção está relacionado à capacidade de produzir INF-y, também devido à lise de células infectadas (Modlin *et. al*., 1983). Em estudos realizados em camundongos, administrando-se anticorpo anti-CD4 e anti-CD8, observou-se o aumento do crescimento bacteriano e conseqüente morte acelerada dos animais, bem como redução na produção de INF-y, o que sugere que ambas as populações de linfócitos são importantes na resistência a *C. pseudotuberculosis*, sendo a imunidade protetora relacionada principalmente à resposta imune celular induzida (Lan *et. al*., 1998).

Adicionalmente, em infecções experimentalmente induzida em ovinos, foi demonstrado através de RT-PCR, o aumento nos níveis de INF-y e TNF- Ŭ, associados às baixas concentrações de IL-4 no local da infecção (Pepin *et. al.*, 1997).

1.6. Diagnóstico

Em pequenos ruminantes, a presença de granulomas externos é altamente sugestiva de linfadenite caseosa, principalmente em rebanhos endêmicos. Quando o material caseoso é devidamente coletado e enviado a um Laboratório de Bacteriologia, é possível identificar facilmente o agente etiológico. Por se tratar de uma doença crônica, o animal que não apresenta sinais clínicos evidentes pode ser um disseminador potencial do microorganismo. O diagnóstico sorológico é uma forma de se identificar os animais infectados e afastá-los do restante do rebanho (Williamson, 2001).

Muitos testes sorológicos para diagnóstico da linfadenite caseosa foram descritos, como a aglutinação, fixação de complemento, hemaglutinação indireta, inibição da hemólise sinérgica, ELISA e *Western Blott* (Ter Laak *et. al*., 1992). A maioria destes testes detecta anticorpos contra exotoxinas de *C. pseudotuberculosis* (Brown & Olander, 1987).

Comparando-se a aglutinação em tubo, fixação de complemento, difusão em gel, inibição da anti-hemolisina e a hemaglutinação indireta, constatou-se que a hemaglutinação indireta era mais confiável e detectava os anticorpos contra a exotoxina por um período maior que os outros testes, apresentando uma vantagem

otuberculosis na sua forma crônica subclínica (sem formação de granulomas em linfonodos externos) (Shigid, 1979).

A inibição da hemólise sinérgica foi desenvolvida para o diagnóstico de *C. pseudotuberculosis* em eqüinos. O teste baseia-se na neutralização provocada pela presença de anticorpos anti-PLD. A reação dos anticorpos com a exotoxina impede que aconteça hemólise sinérgica característica com a toxina de *Rhodococcus equi* em ágar sangue (Knight, 1978). Este teste mostrou-se sensível, embora com pouca especificidade (Brown & Olander, 1987).

Kuria (1989), comparando o teste de inibição de hemólise sinérgica com o ELISA (Enzime-Linked Imunoabsorbent Assay), verificou que este último era muito mais eficiente. Nos testes ELISA tem sido utilizando vários preparados antigênicos como a parede celular bacteriana, que contém um grande número de proteínas imunologicamente ativas, assim como a PLD na sua forma nativa (Sutherland *et. al.*, 1987; Sting *et. al.*, 1998), e obtida através da técnica de DNA recombinante (Menzies *et. al*., 1994) ou antígeno somático preparado a partir do aquecimento da massa bacteriana em uma solução de Sódio Duodecil Sulfato (SDS) com 2-mercaptoetanol (KABA *et. al*., 2001).

Maki e colaboradores (1985), em estudos com ovinos, compararam a PLD nativa e o sonicado de célula como antígenos para o ELISA, constatando-se que o emprego da exotoxina tornava o teste mais sensível. Ter Laak e colaboradores (1992) desenvolveram um teste ELISA sanduíche para detecção de anticorpos contra a PLD em caprinos e ovinos. Foi utilizado como anticorpo de captura, soro hiperimune de coelho contra o sobrenadante de cultura de *C. pseudotuberculosis* em caldo BHI. Os soros com resultados inconclusivos foram submetidos à técnica de *Western Blott*. A especificidade e sensibilidade foram de aproximadamente 100%. Posteriormente, em estudos feitos em rebanhos caprinos e ovinos, este ensaio mostrou-se insuficiente em relação a sua sensibilidade, por apresentar muitos resultados falsos negativos e a necessidade de um aperfeiçoamento da técnica utilizada (Schreuder *et. al.,* 1994; Dercksen *et. al*., 1996).

Em um estudo visando padronizar um teste ELISA para diagnóstico em caprinos, foram utilizados antígenos extraídos por SDS da parede celular da bactéria (antígeno somático) e em paralelo, antígenos obtidos em cultura de 48 horas em caldo BHI, no qual a massa bacteriana foi retirada por centrifugação, seguida por posterior filtração. A sensibilidade e especificidade observada foram de 88% e 77%

ar e 73% e 92% para o antígeno filtrado da cultura (Sting *et. al.*, 1998). Resultados semelhantes foram encontrados por Sutherland e colaboradores (1987), em um experimento com ovinos, no qual se verificou sensibilidade e especificidade de 76% e 73% para antígenos de parede e de 67% e 77% para antígenos secretados em caldo BHI. Os antígenos de parede celular, de acordo com os autores destes trabalhos, revelaram ser mais sensíveis, porém menos específicos que os antígenos secretados.

Derkensen e colaboradores (2000) desenvolveram um teste ELISA sanduíche, baseado no teste ELISA descrito por Ter Laak e colaboradores (1992), para detecção de *C. pseudotuberculosis* em caprinos e ovinos, com o objetivo de melhorar a sensibilidade. Foi utilizado neste ensaio como anticorpo de captura soro hiperimune de coelho contra os antígenos secretados por *C. pseudotuberculosis,* provenientes de cultura em caldo BHI por 72 horas a 37º C. Através deste teste ELISA, constatou-se uma sensibilidade equivalente a 72%, e uma especificidade de 99% em caprinos, e de 51% e 97%, respectivamente, em ovinos.

Kaba e colaboradores (2001) padronizaram um teste ELISA para caprinos, cujo antígeno foi obtido a partir do aquecimento da massa bacteriana com SDS e 2-mecaptoetanol, seguido de centrifugação, utilizando-se o sobrenadante como antígeno. Desta forma, obteve-se uma sensibilidade de 85% e uma especificidade de 96%.

Prescot e colaboradores (2002), em estudos com ovinos, visando detectar a infecção por *C. pseudotuberculosis* através da dosagem de INF-Y utilizando o kit bovigam, constataram uma sensibilidade de 95.7% em 3 ovelhas experimentalmente infectadas e uma especificidade de 95.5%, mas os próprios pesquisadores afirmaram que mais estudos são necessários para definir a sensibilidade e especificidade do ensaio em rabanhos. Menzies *et. al.* (2004) verificaram a aplicabilidade da dosagem de INF-Y, produzido por células do sangue periférico de caprinos estimulados com antígeno de *C. pseudotuberculosis.* Para isso, utilizaram um kit comercial para quantificação de INF-Y bovino e compararam com um ELISA utilizando a PLD recombinante como antígeno. Com um número limitado de animais, no ELISA INF-Y, contatou-se uma sensibilidade de 89.2% e uma especificidade de 97,1%. No ELISA, constatou-se uma sensibilidade de 81% e uma especificidade de 97%. Já Sunil e colaboradores (2008) obtiveram 91% de sensibilidade e 98% de especificidade para ovinos utilizando antígeno inativado com formalina.

tem trazido avanços na prática clínica das doenças infecciosas. A Reação em Cadeia de Polimerase (PCR) é uma técnica altamente sensível, e possui um amplo potencial para detecção de patógenos (Yang & Rothman, 2004), principalmente como diagnóstico complementar por ser um método de alto custo e necessitar de laboratório e equipamentos especializados. Recentemente, foi desenvolvido por Pacheco e colaboradores (2007), um ensaio de multiplex PCR, o qual obteve alta especificidade na detecção de *C. pseudotuberculosis* em cultivo bacteriano oriundo de material caseoso.

Alguns autores questionam apenas o uso de técnicas sorológicas para o diagnóstico da linfadenite caseosa, e defendem o seu uso principalmente para estudos epidemiológicos (Sting *et. al.,* 1998). Entretanto, pesquisadores como Ter Laak e colaboradores (1992) e Dercksen e colaboradores (2000) preconizam o uso dessas técnicas em programa de erradicação da doença, como respaldo para a eliminação de animais com sorologia positiva.

2. JUSTIFICATIVA

Corynebacterium pseudotuberculosis é o agente causador da linfandenite caseosa (LC) em caprinos e ovinos. LC é uma zoonose economicamente importante, que afeta amplamente pequenos ruminantes no mundo, sendo caracterizada pela formação de abscessos nos linfonodos periféricos, e mais raramente nos linfonodos internos e pulmões (Hein & Cargill, 1981). Animais doentes são potenciais reservatórios, sendo que a forma de contágio ocorre principalmente

...do de abscessos superficiais que se rompem nos animais com a forma clínica da doença. Esses abscessos rompidos contaminam camas e abrigos, levando a permanência do microrganismo por longo tempo no ambiente (Yeruham *et. al*., 1997).

No Nordeste do Brasil, a prevalência de linfadenite caseosa nos rebanhos é alta, o que resulta em graves prejuízos econômicos para essa região do país, na qual muito dos pequenos criadores tem a caprinocultura como uma das suas principais atividades produtivas (Ribeiro *et. al.*, 1988). Quase sempre o sistema de criação é ultra-extensivo e não se empregam práticas de reprodução controlada e de vermifugação estratégica. De acordo com Alves and Olander (1999), a desvalorização da pele chega a 40%, ocorrendo também uma significativa diminuição na produção de leite, emagrecimento dos animais, comprometimento de carcaças e gastos com o custeio da mão-de-obra destinada ao tratamento dos granulomas superficiais.

O tratamento da doença é difícil e o uso de antibióticos não constitui uma estratégia de tratamento viável, tendo em vista que as drogas não penetram nos granulomas com facilidade e a drenagem deste leva a contaminação da pele e do ambiente, facilitando a disseminação bacteriana (Nairn & Robertson, 1974). Os granulomas internos são de difícil diagnóstico, podendo constituir focos de disseminação da doença até mesmo para outros rebanhos onde esses animais possam ser introduzidos (Schreuder *et. al*., 1987).

Métodos corriqueiros de diagnóstico e controle da LC não são completamente satisfatórios, embora a vacinação possa reduzir amplamente a infecção (Paton *et. al*., 1995). O melhor método de controle é a erradicação da infecção através da identificação e remoção de animais contaminados (Prescott *et. al.*, 2002). Muitos testes sorológicos vêm sendo descritos (Schreuder *et. al*., 1994; Dercksen *et. al*., 2000), mas com algumas exceções a sensibilidade e a especificidade são inadequadas para produção de um controle confiável baseado na identificação e remoção dos indivíduos contaminados.

Sendo assim, o objetivo desse trabalho foi desenvolver um método diagnóstico sensível, específico e de baixo custo para a correta identificação dos animais infectados com *C. pseudotuberculosis.*

3. HIPÓTESES

- Tendo em vista que o ELISA padronizado para caprinos, utilizando a mesma metodologia utilizada nesse trabalho com pequenas modificações, produziu bons resultados de sensibilidade e especificidade, espera-se então que também seja possível o desenvolvimento de um método de diagnóstico de LC em ovinos, baseado em ELISA, utilizando-se antígenos secretados pela bactéria.

Å Antígenos secretados de *C.pseudotuberculosis* estimularão a produção de INF-y, permitindo a diferenciação de animais não-infectados, animais infectados e animais com sintomatologia clássica.

4.1 Objetivo geral

Desenvolver métodos de diagnóstico sensíveis, específicos e de baixo custo para a correta identificação de caprinos e ovinos infectados com *C. pseudotuberculosis.*

4.2 Objetivos específicos

- Desenvolver um teste ELISA indireto, utilizando como antígeno o sobrenadante da cultura de *C. pseudotuberculosis* em caldo BHI, para detecção de anticorpos específicos em ovinos infectados pela bactéria;

- Determinar a sensibilidade e especificidade deste teste de ELISA para ovinos usando como padrão ouro o isolamento do agente etiológico;

- Comparar o teste de ELISA proposto com outros métodos diagnósticos microbiológicos, moleculares e imunológicos para LC;

- Avaliar a eficácia do ensaio de produção específica de IFN-y em cultura de sangue total estimulada por antígeno secretado de *C. pseudotuberculosis* no diagnóstico da LC em caprinos e ovinos;

- Verificar a sensibilidade e especificidade do ensaio de produção específica de IFN-y em cultura de sangue total estimulada por antígeno secretado de *C. pseudotuberculosis*, em pequenos ruminantes;

- Determinar a capacidade deste ensaio em diferenciar animais não infectados, infectados e sem sintomatologia aparente da doença (subclínicos), e animais infectados e com sintomatologia clássica de LC (clínicos).

5.1 PRIMEIRO MANUSCRITO

Development of an indirect ELISA to identify ovine specific immunoglobulins against *Corynebacterium pseudotuberculosis* employing TH1 stain culture supernatant as antigen.

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Running Title: ELISA for *C. pseudotuberculosis* specific antibodies identification

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Corynebacterium pseudotuberculosis is the etiologic agent of caseous lymphadenitis (CLA), a chronic disease that affects goats and sheep, characterized by granuloma formation in subcutaneous and internal lymph nodes. CLA causes significant economic losses in commercial goat herds; the best procedure to avoid spread of this disease is elimination of infected animals. Secreted antigens from T1 strain bacteria grown in brain heart infusion (BHI) broth were tested in an indirect ELISA system to determine if this system could be used to identify specific immunoglobulins against *C. pseudotuberculosis.* **T**he electrophoretic profile of the BHI antigen was analyzed, as well as the recognition pattern by CLA infected sheep sera. The ELISA results were compared to Multiplex PCR assay and IFN-gamma production. The ELISA was able to discriminate between negative and positive animals, with a sensitivity of 89% and a specificity of 99%, and a large difference between positive and negative optical density values. When this assay was compared with other immunological and molecular methods, it was found only two discrepant results among thirty-two samples. We concluded that an ELISA using secreted antigens from C. pseudotuberculosis T1 strain growth in BHI broth culture would be a reliable tool for the serodiagnosis of caseous ymphadenitis in sheep.

Key words: caseous lymphadenitis, *Corynebacterium pseudotuberculosis,* sheep.

Caseous lymphadenitis is an infectious disease that affects goats and sheep, (Pepin *et. al*., 1999), caused by the facultative intracellular bacteria *Corynebacterium pseudotuberculosis* and is characterized by granulomes in superficial lymph nodes, lungs, liver and spleen. It is an endemic zoonotic disease in the northeast of Brazil, and cuses significant economic losses, due to the mortality of animals and reduction of weight gain and milk production (Unanian, 1989).

Currently, CLA diagnosis in small ruminants is based on characteristic clinical symptoms and on microbiological identification of *C. pseudotuberculosis* in material taken from abcesses. However, efficient control requires serological diagnosis, since infected animals that have no apparent symptoms are a source of infection forr healthy animals (Kaba *et. al*., 2001).

Numerous sorological tests have been developed to detect antibodies against *Corynebacterium pseudotuberculosis* in small ruminants, but no such test has been found satisfactory when applied alone (Paton *et. al*., 1995). Various antigen preparations have been assayed in ELISA tests, including cell wall antigens (Sutherland *et. al*., 1987), phospholipase D exotoxin (ter Laak et. al., 1992; Dercksen *et. al.,* 2000), cell supernatant (Maki *et. al*., 1985), and recombinant exotoxin (Menzies et. al., 1994).

Typically, tests work well for goats (Dercksen *et. al.*, 2000; Kaba *et. al*., 2001), but they have reduced sensitivity in sheep, especially in sheep with subclinically infection that have only internal abscesses. These ELISA assays are not commercially available, and those that are available are relatively expensive (Binns *et. al*., 2007).

In this work, secreted antigens from *C. psedotuberculosis* strain T1 grown in brain heart infusion (BHI) broth culture were tested in an indirect ELISA test for detection CLA in sheep.

MATERIALS AND METHODS

Serum Samples

To test the ELISA assay, it was obtained 70 serum samples from sheep of commerical flocks presenting external lesions typical of caseous lymphadenitis. Caseous matherial was removed, and the etiologic agent was isolated by microbiological technics, as described below. As negative controls, 112 serum samples were obtained from animals without clinical history of caseous lymphadenitis, from a non-endemic area where CLA has been controlled. Samples were stored at -20°C until use.

The ELISA assay was compared with DNA identification and interferon-gamma (INF-y) quantification in other 32 sheep with granulomatous lesions characteristic of CLA. Caseous matherial from these lesions was aseptically collected for bacteriological identification by multiplex PCR, by traditional microbiological technics and Multiplex PCR. Blood was collected in heparinized tubes for interferon-gamma (INF-y) quantification and in tubes without anticoagulant for the ELISA assay.

Serum samples from sheep presenting external lesions with *C. pseudotuberculosis* presence confirmed by microbiological identification (CLA clinically ill animals), from sheep with no clinical sign of the disease, but with positive ELISA results (CLA subclinically infected animals), and from animals from non endemic areas with no clinical history of CLA (negative samples) were tested in a Western blotting system, with the objective to verify the pattern of antigen recognition.

Bacterial Identification

Caseous samples were aseptically collected and plated onto BHI blood agar; the bacteria were identified based on colony morphology, Gram staining, catalase and urease activity, carbohydrate fermentation (glucose, lactose, sucrose and maltose) and synergistic hemolytic activity with CAMP factor of *Rhodococcus equi* (Dercksen *et. al*., 200).

T1 strain secreted antigen production

The antigen was produced as already described by Moura-Costa *et. al. (*2008). Briefly, *C. pseudotuberculosis* T1 strain was cultivated in BHI broth at 37°C for 48h. The culture material was centrifuged for 30 min at 10,000g and the supernatant filtered through a 0.22 εm membrane filter. This supernatant was maintainedt at -20°C until use. Protein concentration was determined by Lowryôs modified method[a]. This antigen was used in the ELISA assay.

Tree-phase partitioning (TPP) Technique for BHI antigen concentration.

The BHI antigen was concentrated by the TPP technique, as alred described by Paule *et. al.* (2004), with the objective to verify its composition in an electrophoresis system. It was analyed by SDS-PAGE electrophoresis and also employed in blood cell culture stimulation. Briefly, 15% ammonium sulfate was added to BHI culture supernatants obtained as described above, and the mixture was gently stirred at room temperature. The pH was adjusted by adding a concentrated HCl solution; then the same volume of n-butanol was added. The mixture was vortexed for 1 minute and allowed to rest for 1h. This mixture was centrifuged at 1350xg for 10 minutes, and the interfacial precipitation was collected and dissolved in a small volume of Tris 20mM, pH 7.4 buffer (500 µl buffer/5 ml culture supernatant). The extracts were dialyzed by ultra filtration with a 10 kDa membrane cut-off[b] against Tris 20mM, pH 7.4. The protein content of the extracts was determined by Lory´s modified method[a].

Polyacrilamide Gel Electrophoresis (SDS-PAGE)

C. pseudotuberculosis secreted antigens were concentrated by Thre-Phase Partitioning (TPP) tecnique (Paule *et. al*., 2004) and separated by one-dimensional polyacrylamide gel electrophoresis under denaturing conditions. A discontinuous SDSï PAGE system with a 4% stacking gel and a 12% running gel was used. The electrophoresis run in 0.124M Tris, 0.96M glycine, and 0.5% SDS, pH 8.3 migration buffer, for 3h at 100V. Each well was loaded with 50µg of protein. Proteins were observed by Coomassie Blue staining.

ELISA for the detection of specific immunoglobulins against *C. pseudotuberculosis*

ELISA was developed based on methodology described by Carminati *et. al.* (2003) used on goats samples. The working dilution of the horseradish peroxidase-conjugated anti sheep IgG immunoglobulin, the optimum protein concentration of antigen solution, and the serum sample dilutions were determined by previous checkerboard titrations, in order to achieve suitable differentiation between positive and negative sera samples. Sera were tested at 1:25; 1:50; 1:100; 1:200; 1:400; and 1:800 dilutions, antigen at 1:400; 1:200; 1:100; 1:50; and 1:25 dilutions, and the secondary antibody was assayed at 1:5,000; 1:10,000; 1:20,000; 1:40,000 and 1:80,000 dilutions (data not shown). The same *C. pseudptuberculosis* positive anda negative control sera samples were used as standards throughout all assays, with the objective to ensure reliability and reproducibility for the results. The antigen, serum and anti-sheep IgG dilution chosen gave a positive/negative optical density (OD) ratio of 12.6.

ELISA microplates[c] were sensitized with BHI C. pseudotuberculosis T1 strain culturesupernatant antigen diluted 1:100 in carbonate-bicarbonate pH 9.6 buffer and incubated at 4^{o}C for 12 hours. The plates were then washed twice with PBS 0.05% Tween 20 (PBST) and blocked with 5% skimmed milk in PBST, and incubated for 2 hours at 37^{o}C. After two additional washes with PBST, 100µl of serum samples diluted 1:100 in PBST 1% skimmed milk were added and the plates were incubated for 1 hour at 37^{o}C. Each serum was tested in duplicate. Plates were washed four times with PBST and a rabbit anti-ovine IgG antibody[d] conjugated with horseradish peroxidase diluted 1:20,000 was added and incubated for 45 minutes at 37^{o}C. After this incubation, the plates were washed and the reaction was developed with 100 εl/well of a solution containing H_2O_2 and tetramehtylbenzidine[e] (TMB) for 15 minutes, and stopped with 4N H_2SO_4. Results were then read in an ELISA plate reader[a] at 450nm.

Western-Blot for the detection of proteins recognized by sera from ovine with clinical caseous lymphadenitis

molecules were separated by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes using 100V for 1 hour; the membranes were temporarily stained with Ponceau´s stain to ensure that the transference process was sucessful. The membranes were blocked with 5% dry skimmed milk in 0.05% PBSï Tween 20 overnight at 4°C. They were then incubated in 1:50 sera from CLA clinically, subclinically ill or soronegative sheep, diluted in PBS-Tween buffer containing 1% dry skimmed milk, for 1h at 37°C, washed five times in 0,05% PBS-Tween buffer, and incubated for 1h with horseradish peroxidase conjugated rabbit anti-sheep immunoglobulins[d] diluted 1:200 in PBSï Tween. Membrane strips were washed five times in 0.05% PBSï Tween and revealed using substrate developer solution (4-chloro-1-naphthol 0.3%, hydrogen peroxide). The reaction was stopped with a final rinse in distilled water and strips were dried and scanned.

Multiplex-PCR for identification of *C. pseudotuberculosis* in caseous material

The Multiplex-PCR was performed as described before (Pacheco *et. al*., 2007). Briefly, bacterial cultures from caseous matherial retrieved from animals with clinical CLA, as described in the previous item, were transferred to a liquid BHI culture medium and incubated for 48h at 37°C. The culture material was centrifugated and the pellet was submitted to DNA isolation, with a commercial kit[f]. The DNA samples were then quantified in a spectrophotometer[a], at 260 and 280 nm.

Multiplex PCRs were performed in a final reaction volume of 10ml containing 1.5UI AccuPrime Taq DNA polymerase[g], PCR Buffer II [200mM Tris/HCl, 500mM KCl, 15mM MgCl2, 2mM dNTPs, 10% glycerol[g]] and 2mM of each of the 16S-F/16S-R, C2700F/C3130R and PLD-F/PLDR2 primers. The template concentration was approximately 30ng of DNA extracted from cultured *C. pseudotuberculosis*. Reactions were carried out in a thermal cycler[h], under the following conditions: initial denaturation at 95°C for 3 min; 40 cycles of 95°C for 1 min, 58°C for 40 s and 68°C for 1 min and 30 s; final extension at 68°C for 7 min. The amplified products were resolved by electrophoresis on 1.0% (w/v) agarose gels and visualized by ethidium bromide staining.

IFN-y quantification

Quantification of INF-y in blood cultures was performed for samples collected from the thirty-two infected sheep as previously described (Paule *et. al*., 2003; Meyer *et. al*., 2005), with some modifications. Briefly, 2h after collection, heparinized blood was incubated at 37°C for 48h in a humidified atmosphere of 5% CO_2 in a 24 well culture tray with 20ε g of antigen concentrated by TPP technique (Paule *et. al*., 2003); 5µl of pokeweed mitogen was used as positive control, and sterile PBS was the negative control. The blood was centrifuged and IFN-y was quantified using an Ovine IFN-y Kit[i]. A cut-off point was calculated using the mean of all not-estimulated cells results, plus three standard deviations. All results above this value (198 pg/ml) were considered significative.

Statistical and mathematical analysis

The cut-off point was calculated as previously described (Frey *et. al*., 1998), with a confidence interval of 99.8%; its corresponds to the mean of all negative sera optical densities (OD) plus three standard deviations.

The sensitivity (S) and specificity (E) and accuracy of the ELISA assay were calculated as follows: S = [TP/(TP + FP)] × 100; E = [TN/(TN + FN)] × 100; A= [(FN + FP/(TP+TN)] × 100. Where TP = the number of truly positive samples, TN = the number of truly negative samples, FP = the number of false-positive samples, and FN = the number of false-negative samples (Presott et. al., 2002).

Repetitivity (RP) was calculated testing the positive and negative controls 40 times each, during the same day and under the same conditions, employing the calculated variation coefficient (CV) found for optical densities values, as follows: RP = (1 ï CV) × 100.

The levels of statistical agreement between diagnostic assays were detected using the Kappa coefficient. The concordance was classified as follows: <0 ï no agreement; 0.19 ï poor agreement; 0.20-0.39 - fair agreement; 0.40-0.59 - moderate agreement; 0.60-0.79 - substantial agreement; and 0.80-1.00 - almost perfect agreement.

In the SDS-PAGE electrophoresis of the BHI antigen, concentrated by the TPP technique (Figure 1), the antigen solution had a complex pattern, with various molecules. Two antigens predominated (130 and 105 KDa). In the Western blot, the BHI antigen was resolved in an SDS-PAGE gel, transferred to a nitrocellulose membrane, and incubated with sera from seronegative animals, from seropositive animals without clinical manifestations of caseous lymphadenitis (CLA subclinically ill animals), and from seropositive animals with external granulomatous lesions with confirmed *C. pseudotuberculosis* (CLA clinically ill animals) (Figure 2). No reaction was seen in strips incubated with sera from negative animals. Sera from seropositve animals without clinical disease gave marked recognition of a 70KDa antigen and discrete recognition of a 32KDa antigen. Animals with clinical manifestations of disease also had antibodies specific for the 70KDa antigens, but they manifested a stronger humoral reaction to the 32Kda antigen.

Table 1 presents the validation criteria for the ELISA assay and the number of sera employed for this standardization. ELISA presented only one false positive result, resulting in a specificity of 99%. However, nine sera from animals with caseous lymphadenitis, confirmed by microbiological assays gave negative results; so the sensitivity was 89%. These results were calculated based on a cut-off point of OD 0.250. Also, the method was quite stable, since repetitivity was 93.2% for the higher values (positive control) and 97.3% for the lower values (negative control).

In the distribution of ELISA results based on OD ranges (Figure 3), the known positive sera gave a normal distribution with peaks within the range of 0.400 to 0.500. Known negative samples were mostly (nearly 70%) below OD 0.100; a small percentage of these negative-sample ODs were near the cut-off (0.250). The mean of the negative samples was 6.5 times lower than the positive samples mean.

Figure 4 shows the distribution of optical densities for the individual animals. There was only one positive result above the cut-off point employed for a seronegative animal. A comparison was made of immunological and molecular techniques for the diagnosis of CLA (Table 2). Multiplex-PCR was able to identify 25 samples as positives. Only two of these samples were negative in the ELISA assay, while three of them demonstrated low and non-significant INF-y production by peripheral blood cells.

agnostic methods results together, one sample (animal ID 1749) was negative for all techniques. Four animals were negative for bacterial DNA identification, but gave positive results in ELISA and IFN-y. Two animals gave discrepant results in the ELISA, when compared to the other methodologies; one was positive in the ELISA, but negative in the other assays, and one was negative in the ELISA, but positive in the IFN-y quantification and bacterial DNA identification.

The Kappa correlation index results calculated in the comparison of the diagnostic assays are shown in Table 3. Only the correlations between IFN-y and PCR, and ELISA and IFN-y quantification gave fair agreement results; all the others were classified as correlations with poor or no agreement.

DISCUSSION

A test with high sensitivity and specificity, at a low cost, would be a valuable tool for the control of CLA, since it would help avoid the introduction of infected animals into a specific region or farm. The main objective of this work was to develop an effective and viable ELISA assay.

First, the antigenic composition of the BHI antigen used in the BHI ELISA was analyzed. The culture supernatant of T1 strain of *C. pseudotuberculosis* has already been analyzed (Paule *et. al*., 2003), and it was they found that serum antibodies of infected goats recognized several antigens, ranging from 16 to 125KDa. Several proteins were found in the *C. pseudotuberculosis* T1 strain culture supernatant, with the 130 and 105KDa bands being the most prevalent (Fig. 1). But when the humoral recognition of these antigens by Western blotting was examined, sera from animals with clinical disease (presenting caseous lesions caused by *C. pseudotuberculosis*) recognized antigens with molecular weights of 70 and 32KDa, while sera from animals without clinical symptoms of CLA, but presenting positive serological results (subclinically infected animals), reacted strongly to the 70KDa antigen, but weakly recognized the 32KDa protein (Fig. 2). It is highly probable that this 32KDa antigen corresponds to the 31.5KDa fraction that has been characterized as the major secreted antigen of *C. pseudotuberculosis*, Phospholipase D (PLD) (Ellis *et. al*., 1991; Costa *et. al*., 1995). Phospholipase D is an exotoxin responsible

ria in the infected animal, and it is a virulence factor related to the formation of the caseous lesion (Hodgson *et. al*., 1992). Consequently, the low level of specific antibodies against this enzyme in animals with no clinical signs of disease is probably due to a low production of the exotoxin by the bacteria or poor humoral immune activation. The 70KDa antigen that was well recognized by both clinical and subclinical animals may be the 67KDa protein described by otherauthors (Paton *et. al*., 1995; Meyer *et. al*, 2004); it is classified as a heat shock protein (Ellis *et. al*., 1991).

Specificity and sensitivity are parameters of major importance in immunodiagnostic; when considered together, they indicate the reliability of the immunologic assay. It was used a large number of ovine serum samples (112 negatives and 70 positives) to establish these parameters, which gave 99% specificity and 89% sensitivity (Table 1). Similar values were already obtained (Prescott *et. al*., 2002), employing the same antigen material in the development of an ELISA test to identify specific antibodies against *C. pseudotuberculosis* in goats; they attained 98.5% specificity and 93.5% sensitivity. Our results demonstrate an advance and an improvement over the ELISA assays developed until now for ovine CLA, since the other scientific reports indicated lower sensitivity. A sonicated somatic antigen was already employed in an ELISA methodology (Binns *et. al*., 2007) , showing 100% specificity and 71% sensitivity. In an attempt to enhance the sensitivity of an ELISA that uses *C. pseudotuberculosis* exotoxin as an antigen (Dercksen *et. al*., 2000), it was developed four different types of ELISA; the highest values obtained for sensitivity and specificity were 79 and 99%, respectively. In this study, microbiological isolation and characterization of the bacteria was used as a gold standard for determining trulypositive animals; but this methodology, besides demanding a long time for its realization, is not applied to subclinical infected animals that have no external caseous lesions.

The cut-off point established for the BHI ELISA was calculated with a methodology described before (Frey *et. al*., 1998), which is a practical and reliable procedure that could be employed since numerous known negative sera samples were available. ROC curve was not applied, since a cut-off point calculated by the Frey *et. al.* (1998) methodology represents an intrinsic statistical and mathematical characteristic of the assay, not submitted to manipulations. We found a repetitivity of 93.2% for the positive controls and 97.2% for the negative controls, with high stability

rs indicate the degree of concordance of results of a sample tested several times.

There were nine animals with clinical symptoms of CLA and with the bacteria confirmed by microbiological assays that presented negative results in the ELISA. These may be false negative results, due to a problem with the sensitivity of this assay, but it must also be considered that some animals have very low production of antibodies against *C. pseudotuberculosis*, especially when the animal expresses a predominantly cellular response against the pathogen. Considering this situation, it was already recommended an IFN-y quantification after specific stimulation of peripheral blood leukocytes (Pepin *et. al*., 1999), in a test complementary to ELISA, in order to identify such animals.

In order to validate our assay, ELISA results were compared with a highly sensitive multiplex PCR (Pacheco *et. al*., 2007) and quantification of IFN-y after stimulation of peripheral blood leukocytes with secreted/excreted antigens of the bacteria. To make this comparison, 32 samples from animals presenting caseous lesions, typical of CLA, were examined. The results were quite satisfactory (Table 2). Only one animal that was identified as positive in mPCR and IFN-y assays gave a negative ELISA result, and it can represent a cross-reaction with some other disease. Also, one animal that was negative in the molecular and cellular immune response assays was positive in the ELISA. Only one animal was negative for all three assays; this may be because of caseous lesions induced by other microorganisms, such as *Arcanobacterium pyogenes* or *Pasteurella multocida* (Dercksen *et. al*., 2000; Dorela *et. al*., 2006). Four animals gave positive results based on ELISA and IFN-y quantification, but were negative for bacterial DNA identification in the granulomatous lesion; this situation could be explained by a previous infection with *C. pseudotuberculosis*; the caseous samples could contain other related bacteria. One animal gave positive results for ELISA, PCR and mPCR, but had low production of IFN-y; this animal may be a low producer of this cytokine (Pepin *et. al*., 1999). The poor agreement found for the correlation of the ELISA assay with PCR and microbiological DNA identification could be explained by the fact that some animals in which *C. pseudotuberculosis* was not identified may had caseous lymphadenitis previously, with development of humoral and cellular responses, identified by ELISA and IFN-y quantification.

CONCLUSIONS

These results demonstrate the reliability and feasibility of using an indirect ELISA, employing T1 strain secreted antigen, as an affordable tool to identify sheep infected by *C. pseudotuberculosis*. This immunodiagnostic assay could be employed in CLA control and eradication programs, with efficient detection of infected animals, and reduced probability of elimination of false-positive animals

SOURCES AND MANUFACTURERS

a. Bio-Rad Laboratories, Hercules, CA.
b. Millipore Corp., Billerica, MA.
c. Corning Life Sciences, Lowell, MA
d. Serotec, Raleigh, NC.
e. Moss Inc., Pasadena, MD.
f. Phoneutria, Belo Horizonte, Brazil.
g. Invitrogen, Carlsbad, CA
h. MJ Research, Waltham, MA
i. MabTech AB, Nacka Strand, Sweden.

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ound for the BHI ELISA developed 1 for identification
2 of specific antibodies against *Corynebacterium pseudotuberculosis.*

PARAMETER	RESULT
Number of tested sera	182
Number of truly positive sera	70
Number of truly negative sera	112
Number of false positives	0
Number of false negatives	9
Cut-Off (Frey Method)	0.250
Sensitivity (%)	89%
Specificity (%)	99%
Repetitivity (positive control)	93.2%
Repetitivity (negative control)	97.3%

Table 2. Comparison between immunologic and molecular assays for caseous lymphadenitis diagnosis employing sera and caseous samples from 32 animals with suggestive clinical symptoms.

Animal ID	Bacterial Identification	Multiplex PCR	Elisa	IFN-gamma
174 c	Neg	Pos	Pos	1095,29
12128	Pos	Pos	Pos	1069,04
107	Pos	Neg	Pos	1005,71
12194	Pos	Pos	Pos	926,13
12212	Neg	Neg	Pos	930,29
12260	Pos	Pos	Pos	670,71
12146	Pos	Pos	Pos	584,04
1749	Neg	Neg	Neg	191,13
1264	Neg	Neg	Pos	47,79
2460	Neg	Pos	Pos	315,71
1727	Neg	Neg	Pos	1102,38
12264	Neg	Pos	Pos	442,79
1375	Pos	Pos	Pos	996,96
11066	Neg	Pos	Pos	895,71
1753	Pos	Pos	Neg	1137,79
1581	Pos	Pos	Pos	1044,88
435	Neg	Neg	Pos	373,21
11028	Neg	Pos	Pos	964,04
551	Neg	Pos	Pos	950,29
105 b as	Neg	Neg	Pos	614,46
44	Pos	Pos	Pos	931,96
1419	Neg	Pos	Pos	998,21
11478	Pos	Pos	Pos	596,13
5096	Neg	Pos	Pos	390,29
12278	Pos	Pos	Pos	736,96
1148	Pos	Pos	Pos	150,71
1068	Neg	Pos	Pos	284,88
1697	Pos	Pos	Pos	1208,63
8162	Neg	Pos	Pos	2458,67
991	Neg	Pos	Pos	2717,56
12238	Neg	Pos	Pos	2340,89
1547	Neg	Pos	Pos	413,11

Table 3. Statistical results for the Kappa's correlation index in the comparison of immunologic and molecular assays employed for the validation of the BHI ELISA assay.

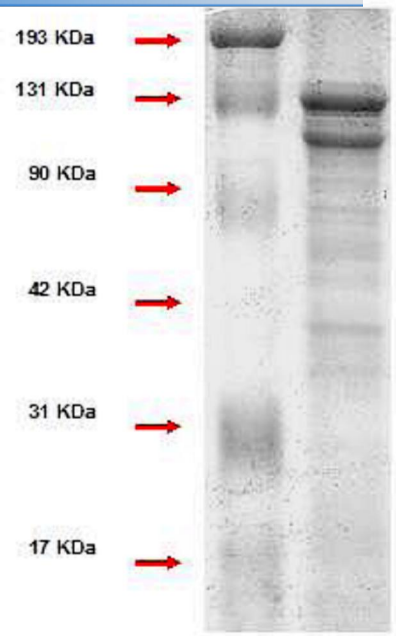
Assays compared	Kappa´s Coefficient	Interpetation
ELISA X IFN-y	0.351	Fair agreement
ELISA X PCR	0.138	No agreement
IFN X PCR	0.309	Fair agreement

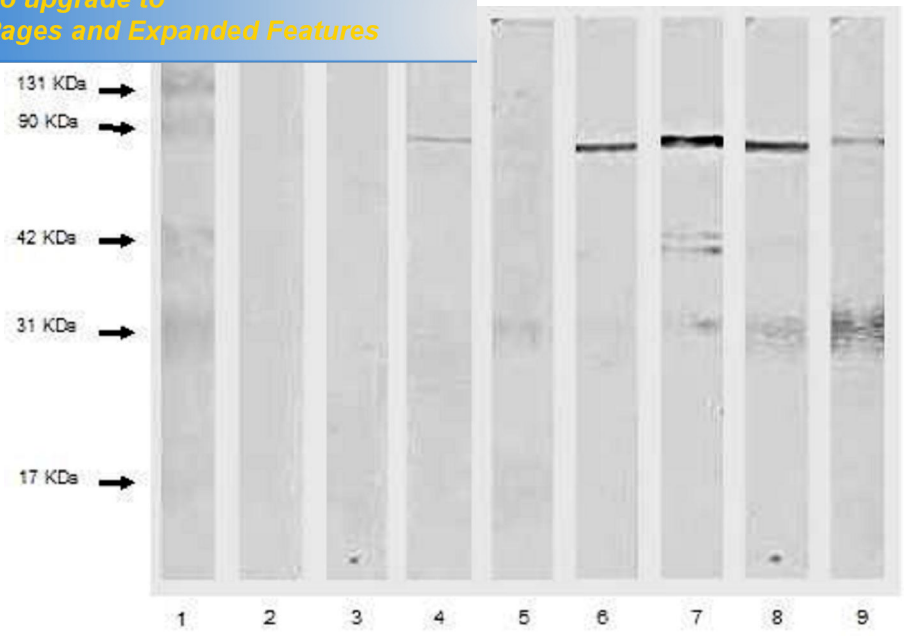
Figure 1. SDS-PAGE electrophoresis profile of *Corynebacterium pseudotuberculosis* antigen from brain heart infusion broth culture, concentrated with the three-phase partitioning technique. (1) Molecular weight standard; (2) *C. pseudotuberculosis* BHI antigen (60 εg). Staining with Coomassie Brilliant Blue. The numbers at left indicate the molecular weight values for the protein standard.

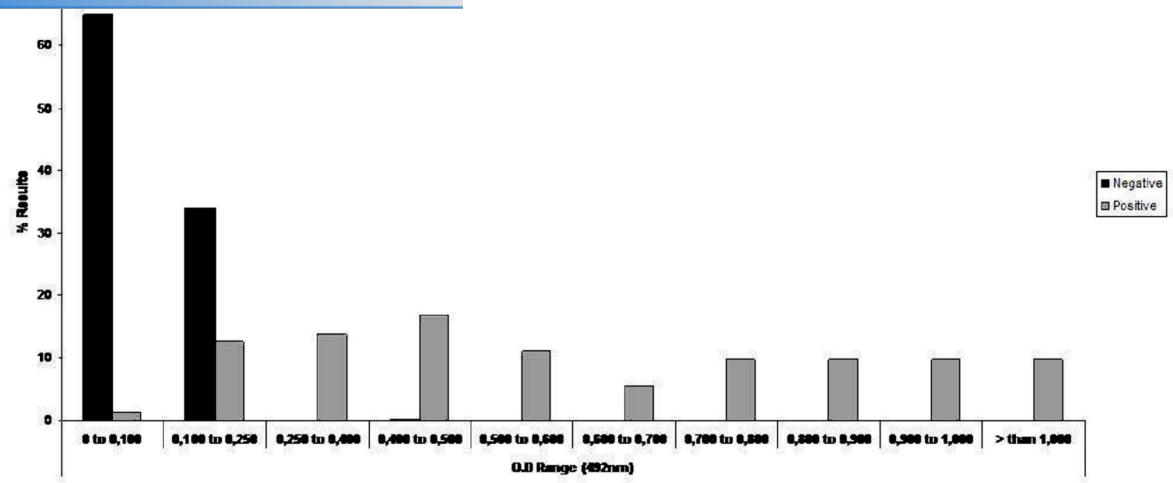
Figure 2. Western-blotting profile of *Corynebacterium pseudotuberculosis* BHI antigen from BHI culture, resolved by SDS-PAGE and incubated with negative ovine sera, sera from sheep with subclinical infection (seropositive by ELISA, but without external symptoms) and samples from animals with clinical caseous lymphadenitis (CLA) (seropositive by ELISA and with caseous lesions). (1) Molecular weight standard; (2) and (3) negative sera; (4), (5) and (6) seropositive animals, without clinical manifestations of CLA; (7), (8) and (9), seropositive animals with caseous lesions. Numbers at left indicate the values of molecular weights from the protein standard, in KDa.

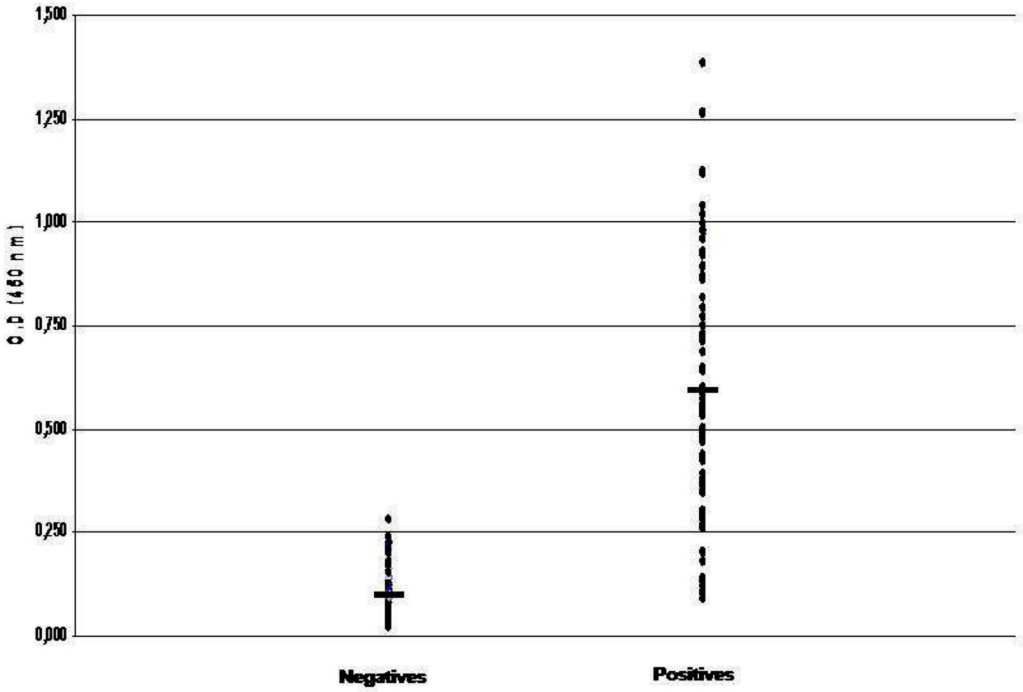
Figure 3. Distribution of the optical densities (OD) results from the BHI ELISA for the detection of specific antibodies against *Corynebacterium pseudotuberculosis*. Data express results obtained with 120 negative sera and 70 positive sera, divided by the percentage in each range. The cut-off point employed was 0.250 OD.

Figure 4. Distribution of the optical densities results obtained in the BHI ELISA for the detection of specific antibodies against *Corynebacterium pseudotuberculosis*, organized by infection status (positive or negative). The data express results obtained with 112 and 70 sera from infected and non-infected animals. The calculated cut-off point was 0.250. Bars indicate the means of optical densities for each group.









Evaluation of IFN-γ production induced by secreted antigens of *Corynebacterium pseudotuberculosis* as a diagnostic marker of caseous lymphadenitis infectious status in sheep and goats.

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Running title: Gamma interferon as caseous lymphadenitis diagnostic marker

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ABSTRACT

ility of the quantification of gamma interferon (IFN-ɔ) levels for the detection of animals infected with *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*) and for determining caseous lymphadenitis (CLA) clinical status was evaluated. Peripheral blood leukocytes were collected from truly negative animals (from non endemic areas and with negative serological results), from seropositive animals for *C. pseudotuberculosis* specific antibodies but without CLA clinical signs, and from seropositive animals with confirmed CLA (each group was composed of thirty sheep and thirty goats). The leukocytes were stimulated with *C. pseudotuberculosis*ï secreted antigens that were concentrated by the three-phase partitioning (TPP) technique. An ovine IFN-ɔ enzyme-linked immunosorbent assay was used to quantify IFN-ɔ production. Goats and sheep with CLA had higher IFN-ɔ levels than uninfected seronegative animals. Leukocytes from sheep with CLA chronic abscesses produced higher IFN-ɔ levels when compared to seropositive sheep without CLA clinical signs. The sensitivity of the assay was 55.8% and 56%, while the specificity was 100% and 93%, for goats and sheep, respectively. In conclusion, IFN-ɔ is a potential marker for the determination of CLA infection status in small ruminants; however, further research is needed to improve assay sensitivity.

Keywords: caseous lymphadenitis; *Corynebacterium pseudotuberculosis;* goat; sheep.

Corynebacterium pseudotuberculosis is the etiologic agent of Caseous Lymphadenitis (CLA) (Brown & Olander, 1987), a disease that is characterized by the formation of chronic abcesses in several organs in small ruminants (Williamson, 2001). Non-apparent infections are common, and failure to diagnose infection in a commercial herd results in high levels of animal-to-animal transmission and significant economic losses (Brow *et. al.*, 1987). The disease is found worldwide and has a severe economic impact on goat and sheep husbandry, leading to decreased milk production, wasting, and condemnation of carcasses due to internal abscesses (Lloyd *et. al*., 1990).

Currently, CLA diagnosis is based on the identification of characteristical clinical signs, represented by the development of the caseous lesions in the lymph nodes and the detection of *C. pseudotuberculosis* in these abscesses (Dorela *et. al*., 2006). Serological diagnostic is of major importance, since subclinically infected animals are a potential source of infection. Many immunological assays have been developed to detect infection, the majority of which detect the humoral antibody response to infection (Ter Laak *et. al.,* 1992; Sting *et. al*., 1998; Dercksen *et. al*., 2000; Kaba *et. al*., 2001; Carminati *et. al*., 2003; Binns *et. al.*, 2007), with varying levels of success.

Sensitivity and specificity are important factors to be considered while making the choice of diagnostic assay(s) for a screening program. A test with a lower specificity can lead to false-positives, while a reduced sensitivity can lead to false-negatives results. The presence of infected animals in a herd may represent a source of environmental contamination. Problems with sensitivity and specificity in immunodiagnostic assay can be due to many factors, especially in animals with a low or undetectable humoral response against *C. pseudotuberculosis* (Binns *et. al*., 2007), and cross-reactivity with other related microorganisms (Williamson, 2001; Dorella *et. al*., 2006), respectively.

Since *C. pseudotuberculosis* is a facultative intracellular pathogen, cell-mediated immunity is an important component of the protective immune response (Lan *et. al*., 1998). An alternative approach to the immunodiagnosis of CLA is the assestment of the cellular immune response to *C. pseudotuberculosis* antigens

et. al., 2004; Meyer *et. al*., 2005; Sunil *et. al.,* 2007), and this can be carried out in association with serological assays. Previously, a whole-blood gamma interferon (IFN-y) assay was found to be a promising detection tool for the eradication of CLA from small ruminant flocks (Menzies *et. al*., 2004; Meyer *et. al*., 2005; Sunil *et. al*., 2007)

In this study, quantification of IFN-y induced in leukocites stimulated with secreted-excreted antigens derived from *C. pseudotuberculosis* T1 straim was evolueted. The objective of thi work was to determine its utility for the detection of *C. pseudotuberculosis* infected goats and sheep and for differentiating between subclinical and clinical CLA.

MATERIALS AND METHODS

Serum and whole blood samples

In order to compare IFN-ɔ production in leukocytes from animals with different states of infectivity and clinical status, 5 ml of serum and heparinized whole blood were collected from goats and sheep which were divided into three groups for each species: (1) Non-infected group: healthy animals from a non-endemic area with no evidence of CLA and that were seronegative in an enzyme-linked immunosorbent assay (ELISA); (2) Infected-subclinical CLA group: seropositive animals without clinical symptoms of the disease; and (3) Infected-clinical CLA group: seropositive animals presenting with clinical symptoms of CLA and from which *C. pseudotuberculosis* was isolated from the lesions. Each group was composed of thirty goats and thirty sheep. For infectivity status analysis, group 1 was named as non-infected animals (n=30 for each species, negative in ELISA), and groups 2 and 3 were assembled and considered infected animals (n=60 for each species, all animals positive in ELISA).

To evaluate the IFN-ɔ assay as a diagnostic test and to perform a validation study, 57 blood and serum samples (5 ml each) from seropositive goats with caseous lesions, and with bacterial infection confirmed by bacteriological assays (described later) and Multiplex PCR (Pacheco *et. al*., 2007) (named truly positive) were screened. Also, 30 samples from seronegative animals from a non endemic area

d. Using the same criteria, 42 truly negative and 42 truly positive samples from sheep were also obtained.

Bacterial identification

Caseous samples from clinically ill animals (collected from enlarged superficial lymph nodes, one from each animal) were aseptically collected and plated onto brain heart infusion (BHI) agar supplemented with 5% defibrinated sheep blood. Plates were incubated aerobically for approximately 48h at 37°C. Colonies morphologically resembling *C. pseudotuberculosis* were Gram stained. Gram positive colonies were further tested for urease activity, synergistic hemolytic activity with Christie, Atkins, Munch-Peterson (CAMP) factor from *Rhodococcus equi* and carbohydrate fermentation (glucose, lactose, sucrose) (Dercksen *et. al*., 2000) . Strains that were positive for urease and glucose fermentation, negative for lactose and sucrose fermentation and enhanced the hemolysis of *R. equi* were considered to be *C. pseudotuberculosis*. To further characterize the caseous samples retrieved; a multiplex PCR assay was performed as previously described by Pacheco *et. al*. (2007), with the objective to provide a more sensitive determination of the presence of *C. pseudotuberculosis* in infected animals.

The preparation of the *C. pseudotuberculosis* secreted antigen was carried out as previously described by Moura-Costa *et. al*. (2008). Briefly, the *C. pseudotuberculosis* T1 strain was cultivated in BHI broth at 37°C for 72 hr. The culture was centrifuged for 30 min at 10,000 × *g*. The supernatant was filtered through a 0.22-ε m membrane filter,[a] and was then kept at ï 20°C until use. The quantification of protein was determined by Lowryôs modified method.[b]

***C. pseudotuberculosis* secreted antigen production for ELISA**

The preparation of the *C. pseudotuberculosis* secreted antigen was carried out as previously described. By Moura-Costa *et. al*. (2008). Briefly, the *C. pseudotuberculosis* T1 strain was cultivated in BHI broth at 37°C for 72 hr. The culture was centrifuged for 30 min at 10,000 × *g*. The supernatant was filtered through a 0.22-ε m membrane filter,[a] and was then kept at ï 20°C until use. The quantification of protein was determined by Lowryôs modified method.[b]

C. pseudotuberculosis secreted antigen concentration

The C. pseudotuberculosis antigen preparation was concentrated using the three-phase partitioning (TPP) techinique as described by Paule *et. al*. (2004). Briefly, 15% of ammonium sulfate was added to the culture supernatants at room temperature and the mixture was stirred gently. The pH was adjusted by the addition of HCl and the same volume of n-butanol was added. The mixture was vortexed for 1 minute and allowed to rest for 1h. The mixture was centrifuged at 1350xg for 10 minutes and the interface precipitate was collected and dissolved in Tris (20mM, pH 7.4) buffer (500 µl buffer/5 ml culture supernatant). Extracts were dialyzed by ultrafiltration with a 10kDa membrane cut-off[a] against Tris 20mM, pH 7.4. The protein content of the extracts was determined by Lowry´s modified method (Peterson, 1979).[b]

ELISA for the detection of specific immunoglobulins against _C. pseudotuberculosis_

The ELISA was carried out as described by Prescott *et. al.* (2000) and Carminati *et. al.* (2003), with minor modifications. The ELISA microplates[c] were coated with BHI-culture antigen, diluted 1:100 in carbonate-bocarbonate buffer (pH 9.6), and incubated at 4°C for 12 hours. The plates were then washed twice with phosphate buffered saline, 0.05% Tween 20 (PBST), blocked with 5% skimmed milk in PBST, and incubated for 2 hours at 37°C. After 2 additional washes with PBST, 100µl of sheep or goat serum samples, diluted 1:100 in PBST with 1% skimmed milk were added, and the mixture was incubated for 1 hour at 37°C. Each serum sample was tested in duplicate. Plates were washed 4 times with PBST. Rabbit anti-ovine Immunoglobulin G (IgG) antibody[d] or donkey anti-goat IgG conjugated with horseradish peroxidase,[e] diluted 1:20,000 and 1:10,000, respectively, in PBST, was added, and the mixture was incubated 45 minutes at 37°C. After incubation, the plates were washed and reaction was developed with tetramehtylbenzidine[f] chromogen and hydrogen peroxidase substrate (100µl/well) for 15 minutes and the reaction was stopped with 4N H_2SO_4. Results were read in an ELISA plate reader[b] at 450nm.

ining the assay with the higher optical density (OD) value for the positive control, the lowest OD for the negative control and the lowest OD result for the blank (with no sera addition), as the standard plate. A correction factor was developed dividing the positive control OD value of the standard plate by the positive control OD value found in the assayed plate, and all serum samples OD values of this same plate were corrected by this factor.The same negative and positive controls were used in all plates. The cut-off values for the ELISA were applied as described before by Prescott *et. al*. (2000) and Carminati *et. al.* (2003).

Gamma interferon quantification

Quantification of INF-y in blood cultures was performed in samples collected from experimental animal groups, as previously described (Paule *et. al*., 2003; Meyer *et. al*., 2005), with some modifications. Briefly, 2h after collection, 1 ml of heparinized blood was incubated at 37°C for 48h in a humidified 5% CO_2 atmosphere in a 24-well culture tray with 20 ε g TPP antigen (Paule *et. al*., 2003), 5 µl of pokeweed mitogen (1 ε g/ml) as positive control, or Phosphate Buffered Saline (PBS) as a negative control. After this period, the blood was centrifuged and IFN-y was quantified in the supernatant, using an Ovine IFN-y Kit[i].

Statistical and mathematical analysis

The comparison between the IFN-ɔ quantification results from non-infected animals and seropositive animals, as well between not-infected, CLA subclinical and clinically infected animals was carried out using the Mannï Whitney nonparametric test at the SPSS v13.0 softwareh , and a p value < 0.05 was considered significant.

The cut-off point for IFN-ɔ quantification was calculated as previously described (Frey *et. al*., 1998), with a confidence interval of 99.8%, corresponding to the mean of the negative sera optical densities, plus 3 standard deviations. Fifty-seven blood samples from seropositive goats with caseous lesions and confirmed bacterial infection (truly positive group) and 30 samples from seronegative goats from a non-endemic area (truly negative group) were evaluated. Using the same criteria, 42 truly negative and 42 truly positive sheep samples were evaluated.

pecificity (E) of the IFN-ɔ assay were calculated as follows: S = (TP/ (TP + FP)) × 100; E = (TN/ (TN + FN)) × 100. Where TP = the number of truly positive samples, TN = the number of truly negative samples, FP = the number of false-positive samples, and FN = the number of false-negative samples (Prescott *et. al*, 2002).

RESULTS

This study had the objective to evaluate the specific interferon-gamma production by peripheral blood leukocytes stimulated with *Corynebacterium pseudotuberculosis* secreted antigen, and its application as a diagnostic marker for caseous lymphadenitis in sheep and goats.

Pokeweed mitogen (used in the experiment as a stimulation positive control) induced IFN-ɔ production by peripheral blood cells in both seronegative and seropositive goats and sheep, without significant statistical difference between groups. There were significant differences in the IFN-ɔ levels between seronegative and seropositive goats (Mannï Whitney test) (Fig. 1a), as well as for sheep that were negative or positive for the presence of *C. pseudotuberculosis* specific antibodies (Fig. 1b), showing that IFN-ɔ quantification was able to differentiate seronegative and seropositive groups.

When the IFN-ɔ quantification in samples from goats with different clinical status of CLA was examined, there was a significant difference in induced IFN-ɔ levels between uninfected animals and CLA subclinically and clinically infected animals (Fig. 2a); however, the levels in goats with a subclinical infection were not significantly different to animals with *C. pseudotuberculosis* clinical infection. In contrast, sheep with CLA clinical signs produced significantly more IFN-ɔ than subclinically infected animals and non-infected animals. However, there was no statistically significant difference in IFN-ɔ levels between the subclinical group and the non-infected group (Fig. 2b).

The IFN-ɔ production in sheep and goats was compared in order to determine if the cellular response to *C. pseudotuberculosis* antigens differed between these two species (Fig. 3). However, no significant differences in IFN-ɔ levels were observed between these species, regardless of CLA clinical status.

ave a significant correlation with the amount of IFN-ɔ produced by peripheral blood cells after stimulation with the *C. pseudotuberculosis* antigens in the seropositive and clinically infected goats and sheep (r = 0.027 and 0.0204, Fig. 4a and 4b, respectively). The distributions of the individual results obtained for IFN-ɔ levels in goats and sheep are shown in Figures 5a and 5b, respectively. There was no positive result for seronegative goats above the cut-off (204 pg/ml). However, the cellular response of seropositive animals was more variable, with a broad range between the maximum and the minimum results. A similar response pattern was observed in seropositive sheep. Furthermore, some sheep exhibited significant IFN-ɔ production even though they were seronegative.

The determination of specific IFN-ɔ levels following antigen stimulation could be used as a diagnostic assay. Consequently, validation parameters were observed to determine if this assay could be used for indicating *C. pseudotuberculosis* infectivity status in sheep and goats. Consequently, a specificity of 100% and a sensitivity of 55.8% were observed for goats, using a cut-off of 204 pg/ml. Out of a total of 87 samples, no false positives were found, although there we identified 45 false negatives (Table 1).

The same procedure was used to evaluate sheep (Table 2). Of 84 samples, three presented false-positive results, and 33 were false negatives. The sensitivity and specificity of the assay were 56% and 93%, respectively, and the cut-off point was calculated to be 508 pg/ml.

DISCUSSION

Several aspects of *C. pseudotuberculosis* infection in sheep and goats have been wide studied due to the importance of CLA in commercial herds. However, although some information concerning cellular and humoral responses to this bacterium is now available, few studies have focused on the development of reliable diagnostic and immunoprophylaxis tools (Menzieis *et. al*., 1994; Prescott *et. al*., 2002; Carrminati *et. al*., 2003; Binns *et. al*., 2007). The applicability of IFN-y quantification after stimulus of peripheral blood leukocytes with TPP-concentrated secreted C. pseudotuberculosis antigen was examined and the efficacy of this

etermination of infection and clinical CLA status in sheep and goats.

The TPP-concentrated secreted antigen from the T1 strain of *C. pseudotuberculosis* was reported to cause significant stimulation of leukocytes from peripheral blood collected from small ruminants (Paule *et. al*., 2004). Employment of this assay in goats after stimulation with secreted and somatic antigens from *C. pseudotuberculosis* has also been described (Meyer *et. al*., 2004). Secreted antigens in a chemically defined medium (Moura-Costa *et. al*., 2008) presented an increased stimulation, with higher production of IFN-ɔ, when compared to the stimulation induced by somatic antigen. However, the weak induction of IFN-ɔ by somatic antigen was not observed by other researchers (Menzieis *et. al*, 1994; Pepin *et. al*., 1997; Pollock & Andersen, 1997); this discrepancy can be explained by the utilization of different pathogenic, attenuated, or genetically modified strains (Paule *et. al*., 2004), or because of different antigen purification methods. Some researchers (Sunil *et. al*., 2008) were not able to obtain significant results when a somatic antigenic lysate was used to stimulate leukocytes collected from re-infected sheep; better results were observed when peripheral blood cells were stimulated with formalin-inactivated bacteria.

In the present study, when seronegative and seropositive animals were tested to look for differences in IFN-ɔ levels, significant differences were found in both sheep and goats (Figures 1a and 1b). However, this response was extremely variable, especially when individual results were analyzed (Figures 5a and 5b). Similar diverse cellular responses to secreted antigens from *C. pseudotuberculosis*, in both naturally and experimentally infected animals, have been reported previously (Moura-Costa *et. al*., 2008).

IFN-ɔ levels were also investigated in animals presenting different CLA clinical status. Goats with subclinical or clinical CLA could be distinguished from negative cases in the current study, as could sheep with clinical symptoms from healthy animals. Subclinically infected sheep presented a significantly lower IFN-ɔ production compared to animals with clinical disease (Figures 2a and 2b). The importance of IFN-ɔ for protection against progression of this disease and as a major intermediate in the cellular response against this microorganism has been previously reported (Lan *et. al*., 1998; Pepin *et. al*., 1997). However, results of the current study contradict the hypothesis that some animals with clinical lesions can have decreased

rial antigens and those seropositive animals with no apparent symptoms of disease can produce high levels of IFN-ɔ, resulting in protection against the pathogenic mechanisms of CLA (Meyer *et. al*., 2004).

The sensitivity and specificity values obtained in the current study were 55.8% and 100% for goats (Table 1) and 56% and 93% for sheep (Table 2), respectively. It was found that the IFN-ɔ quantification assay presented herein has characteristics similar to several serological assays that detect specific *C. pseudotuberculosis* antibodies; however, the main problem observed in the majority of these assays is their low immunodiagnostic sensitivity (Sutherland *et. al*, 1987; Menzieis *et. al*., 1994; Sting *et. al*., 1998). There were large differences in the intensity of the cellular response elicited by the bacteria. Whether this discrepancy was caused by different routes of infection, persistence of the microorganism in the animal, differences in distribution and dissemination, the genetic background of the species or breeds, or the bacterial strain involved, is not currently understood. In the case of *Mycobacterium tuberculosis*, a microorganism that is phylogenetically related to *C. pseudotuberculosis*, experimentally infected bovines were reported to develop a highly variable cellular immune response against culture filtrate antigens derived from the bacteria and the response was reported to vary depending on the stage of infection (Prescott *et. al*., 2002).

Sensitivity is a major validation parameter in a diagnostic assay that is being considered for use in eradication programs, principally to ensure that no infected animals are misdiagnosed, since such animals can be important sources of infection (Kaba *et. al.,* 2001). High values of sensitivity (91%) for IFN-ɔ quantification were reported in an assay using formalin-inactivated somatic antigenstimulated sheep derived cells (Sunil *et. al*., 2008); however, only 3 infected animals were evaluated and the calculation of sensitivity and specificity was based on 33 observations on these animals. The study also reported that the IFN-ɔ assay failed to give consistent results over time.

The standardization of an IFN-ɔ assay, using secreted *C. pseudotuberculosis* antigens, has been described previously (Meyer *et. al*., 2004). A somatic antigenic lysate and secreted antigens were used, and it was concluded that secreted antigens were better for stimulating goat blood cells. In the current study, a larger number of animals were used with the objective to test this assay as a diagnostic tool. The main

he poor sensitivity of the assay; but the specificity obtained for sheep and goats was high (Tables 1 and 2).

In mice and humans, IFN-ɔ is part of the cellular immune response, directing the response pattern towards a Th1 profile (Mosmann *et. al*., 1986; Tsicopoulos *et. al*, 1992). IFN-ɔ also plays an important role in the humoral response, particularly in isotype switching, inhibiting changes that are IL-4 dependent and inducing production of certain IgG subclasses (Abbas *et. al*., 2008). However, in sheep and goats, the conditions that induce a Th1 or Th2 profile are not well understood and little is known about isotype switching in B lymphocytes. Taking this into consideration, the correlation between specific IFN-ɔ levels and the presence of serum antibodies specific for the antigen used for blood cell stimulation were examined in the present study. A significant correlation between IFN-ɔ levels and antibody production in animals presenting clinical symptoms of the disease was not found (Figures 4a and 4b), but this conclusion applies only to this disease as it cannot be extended to the small ruminant immune response in general.

In the current study, a TPP-concentrated secreted antigen was employed. The preparation of this antigen is very simple, relatively fast and has low production costs. This antigenic formulation represents an inexpensive and accessible method for use in the IFN-ɔ quantification assay. The sensitivity of this method requires further development, including improvements in blood cell culture conditions, antigen extraction, and stimulation protocols, as well as the sandwich ELISA for IFN-ɔ quantification in order to enhance its sensitivity.

Sources and manufacturers

a. Millipore Corp., Billerica, MA.
b. Bio-Rad Laboratories, Hercules, CA.
c. NuncÊ , Thermo Fisher Scientific, Rochester, NY.
d. Bethyl Laboratories Inc., Montgomery, TX.
e. Dako North America Inc., Carpinteria, CA.
f. Moss Inc., Pasadena, MD.
g. MabTech AB, Nacka Strand, Sweden.
h. IBM SPSS Statistics, Chicago, IL.

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Table 1. Validation parameters for the gamma interferon assay in goats, obtained using 87 sera and whole-blood samples.

PARAMETER	RESULT
Total number of tested samples	87
Number of truly positive samples	57
Number of truly negative samples	30
Numer of false positives	0
Number of false negative samples	45
Cut-Off (Frey Method)	204 pg/mL
Sensibility (%)	55.8
Specificity (%)	100

r the gamma interferon assay in sheep, obtained using 84 sera and whole-blood samples.

PARAMETER	RESULT
Total number of tested samples	84
Number of truly negative samples	42
Number of truly positive samples	42
Number of false positives	3
Number of false negatives	33
Cut-Off (Frey Method)	508 pg/mL
Sensibility (%)	56
Specificity (%)	93

Figure 1. Peripheral blood leukocyte production of gamma interferon in goats **(a)** and sheep **(b)** after stimulation with a pokeweed mitogen (PWM) or three-phase partitioning (TPP) concentrated secreted and/or excreted *C. pseudotuberculosis* antigens and in the absence of stimulation (control). Animals were classified by infectivity status. Sixty seropositive and thirty seronegative animals were tested twice in the experiment. Results represent the means of each group, and bars indicate the standard deviation. An asterisk (*) indicates a significant difference based on the Mannï Whitney test ($p < 0.05$) between seronegative and seropositive animals in each group.

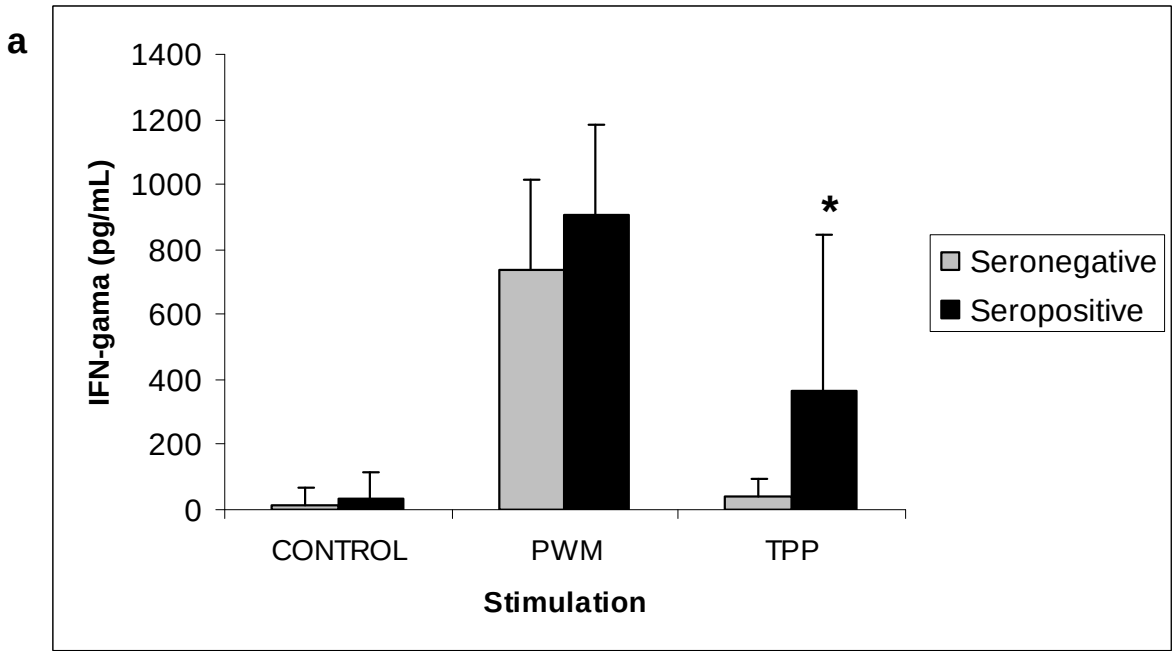
Figure 2. Quantification of IFN-ɔ produced by peripheral leukocytes stimulated with PWM, three-phase partitioning (TPP-concentrated secreted and/or excreted antigens, and in the absence of stimulation (control). Results for blood samples retrieved from goats (a) and sheep (b) are presented. Animals were divided by CLA clinical status, and 30 samples were tested in each group, for both sheep and goat samples. Results represent the means of each group, and the bars indicate the standard deviation. An asterisk (*) indicates a significant difference between the clinical or subclinical groups and the control group, and two asterisks (**) indicate a significant difference between clinical and subclinical animals, based on the Mannï Whitney test ($p < 0.05$).

Figure 3. Gamma interferon production in non-infected, subclinically and clinically infected sheep and goats, following stimulation with secreted and/or excreted *C. pseudotuberculosis* antigens. Thirty goats and sheep were tested in each group. Results represent the means of each group and the bars indicate the standard deviations. No statistical difference was observed between the goats and sheep in any of the groups studied.

Figure 4. Correlation between the ELISA optical density (OD) 1 results for the detection of specific antibodies against *C. pseudotuberculosis* and gamma interferon levels after stimulation with TPPï concentrated antigen in goats **(a)** and

0 sheep) seropositive animals presenting with CLA symptoms were tested. The correlation index was r = 0.027 and r = 0.0204 for goats and sheep, respectively.

Figure 5. Distribution of the individual results obtained for gamma interferon levels in the supernatant of peripheral blood culture after stimulus with TPPï concentrated antigen in goats **(a)** and sheep **(b)**. The gray line indicates the cut-off point calculated for the assays (goats - 204 pg/ml; sheep ï 508 pg/ml).



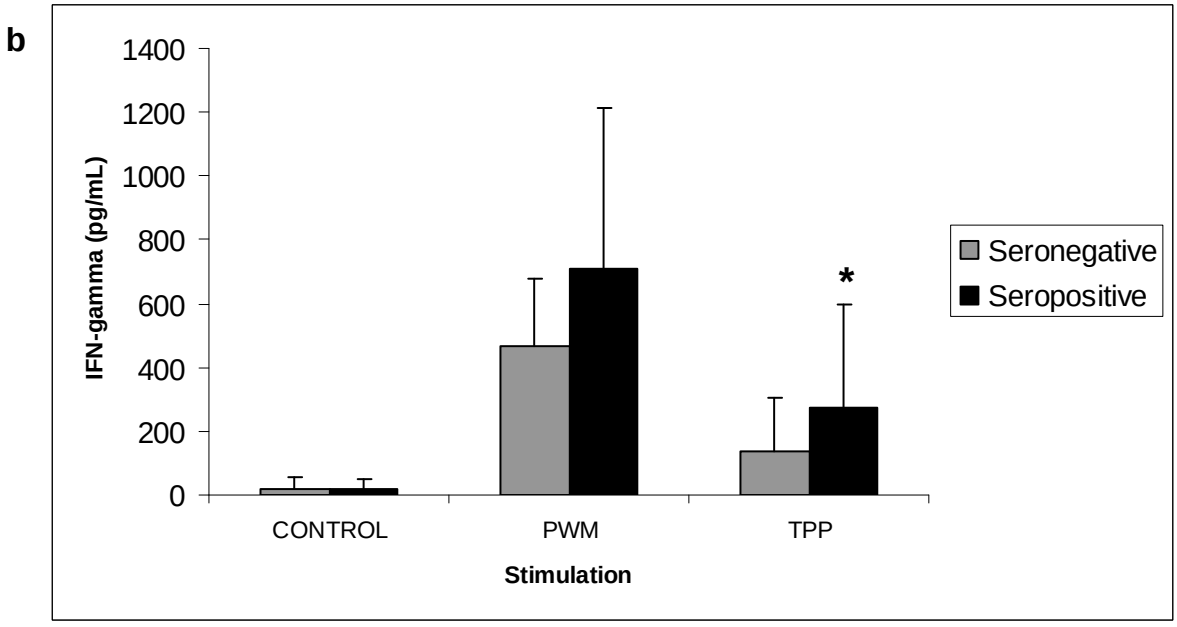


Figure 1- Peripherical blood leukocyte production of IFN-y in goats **(a)** and sheep **(b),** after stimulation with pokeweed mitogen (PWM) or Three-phase partitioning (TPP) concentrated secreted and/or excreted antigen, and in the absence of stimulation (control). Animals were classified by its infectivity status. Sixty seropositive and thirty seronegative animals were tested twice in the experiment. Results are the means for each group, and bars indicate the standard deviation. An asterisk (*) indicates significant difference based on the Mann-Whitney test ($p<0.05$) between seronegative and seropositive animals in each group.

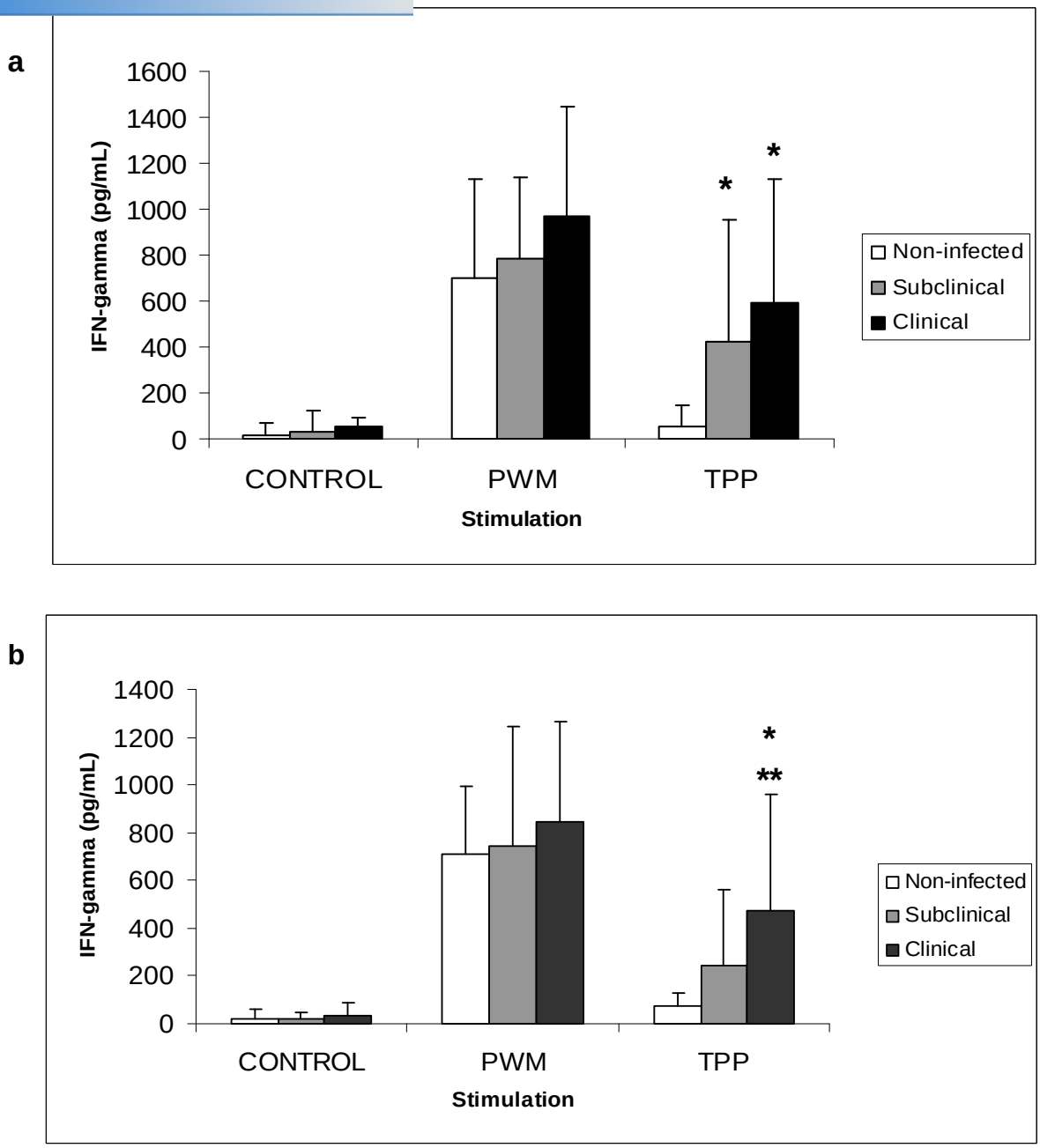


Figure 2- Quantification of IFNɔ produced by peripheral leukocytes stimulated with a pokeweed mitogen (PWM), three-phase partitioning (TPP)-concentrated secreted and/or excreted antigen, and in the absence of stimulation (control). Results for blood samples retrieved from goats (a) and sheep (b).Animals were divided by caseous lymphadenitis clinical status, and 30 samples were tested in each group, for each species. Results are the means for each group, and bars indicate the standard deviation. An asterisk (*) indicates a significant difference between clinical or subclinical groups and the control group, and two asterisks (**) indicate a significant difference between clinical and subclinical animals, based on the Mannï Whitney test ($p < 0.05$).

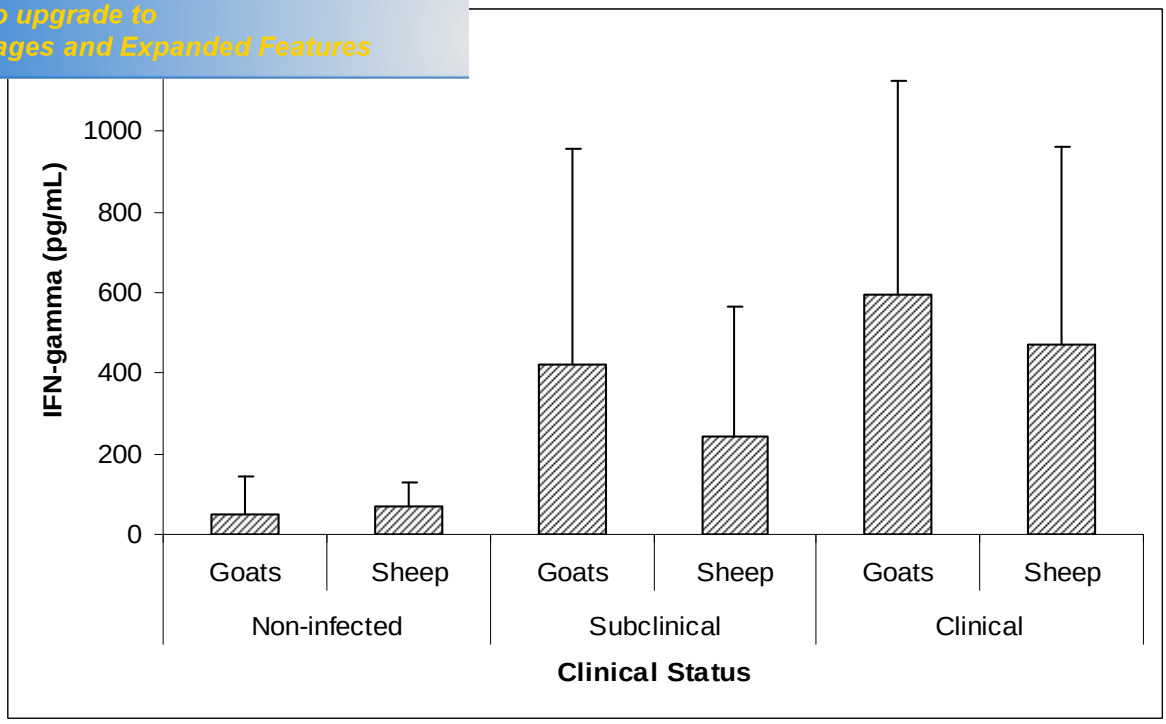
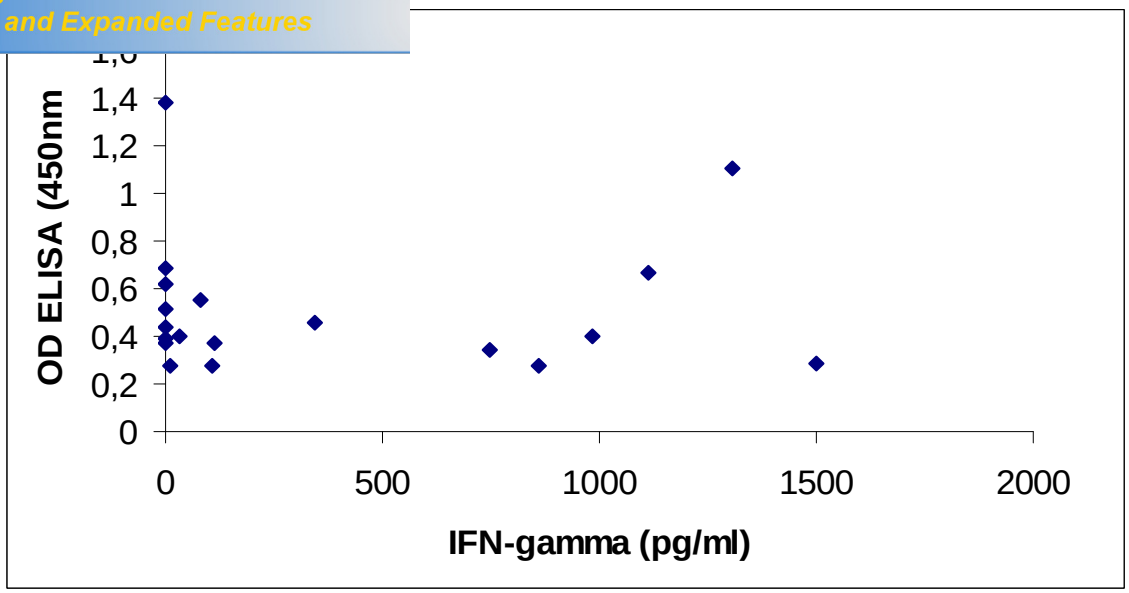


Figure 3- Gamma interferon production non-infected, CLA subclinical and CLA clinical sheep and goats, after stimulation with secreted and/or excreted antigens of *Corynebacterium pseudotuberculosis*. Thirty goats and sheep were tested in each group. Results are the means for each group, and bars indicate the standard deviations. No statistical difference was found between goats and sheep in any group.



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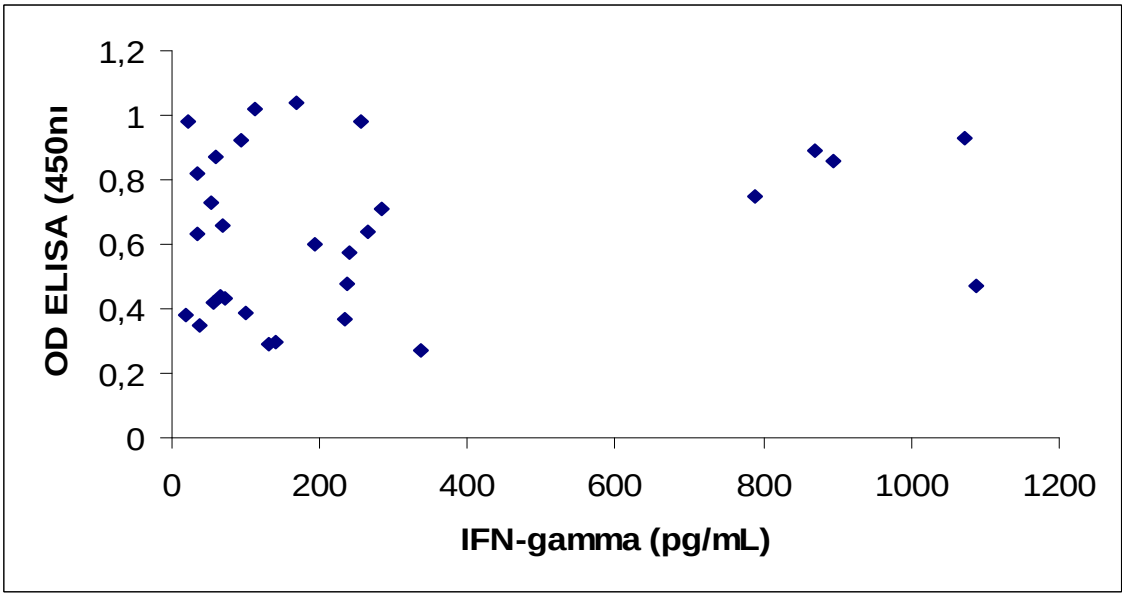
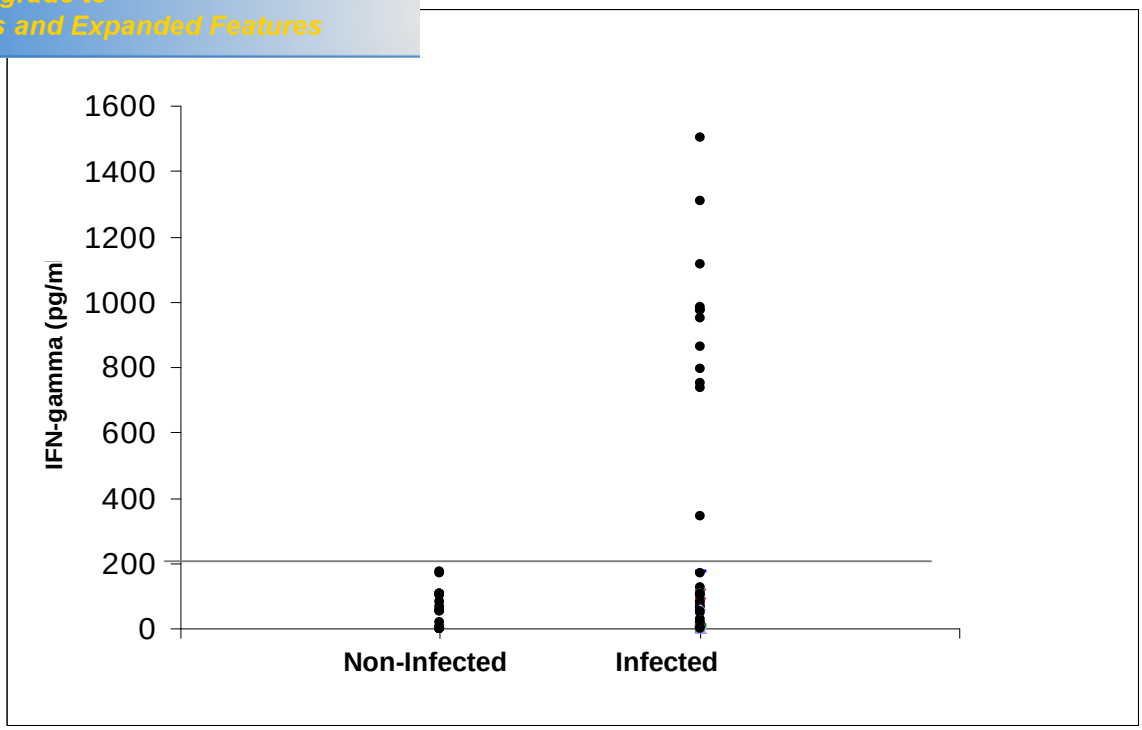


Figure 4- Correlation between the ELISA optical density (OD) results for the detection of specific antibodies against *C. pseudotuberculosis* and gamma interferon levels after stimulation with TPPï concentrated antigen in goats (a) and sheep (b). Sixty (30 goats and 30 sheep) seropositive animals presenting with CLA symptoms were tested. The correlation index was r = 0.027 and r = 0.0204 for goats and sheep, respectively.



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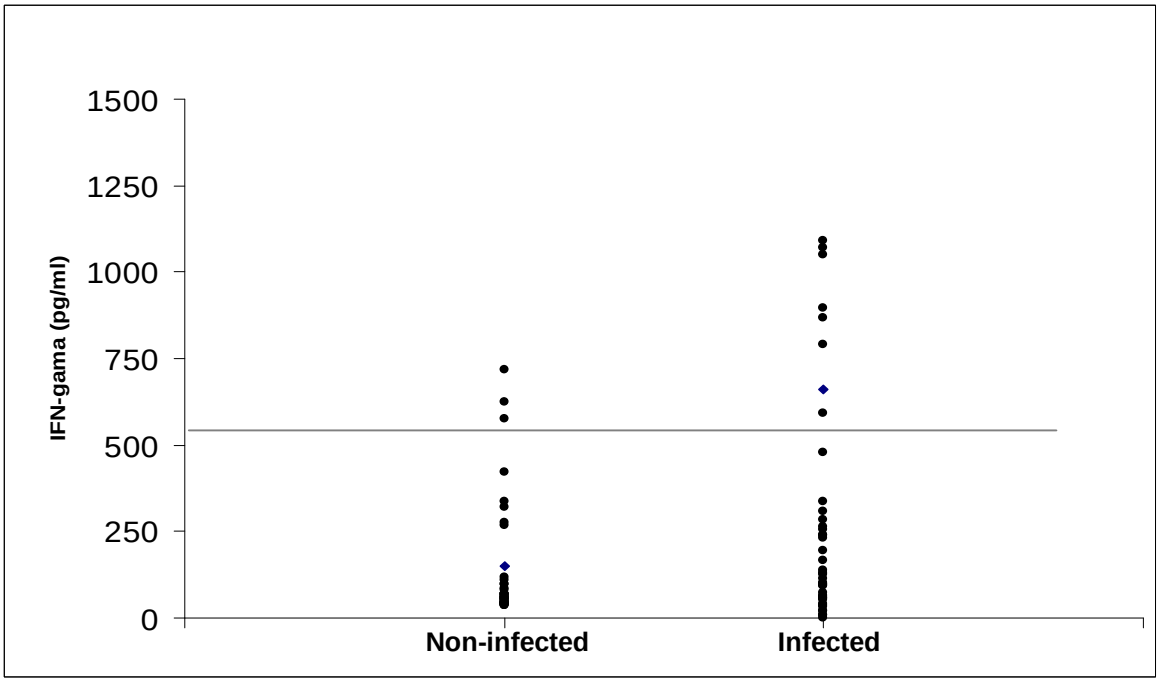

Figura 5- Distribution of the individual results obtained for gamma interferon levels in the supernatant of peripheral blood culture after stimulus with TPPï concentrated antigen in goats (a) and sheep (b). The gray line indicates the cut-off point calculated for the assays (goats ï 204 pg/ml; sheep ï 508 pg/ml).

6. CONCLUSÕES

- A sensibilidade e especificidade do teste ELISA desenvolvido com a fração secretada de *C. pseudotuberculosis* para diagnóstico da linfadenite caseosa em ovinos foram de 89% e 99% respectivamente, mostrando-se como um teste satisfatório para a detecção de animais infectados pela bactéria.

- O antígeno utilizado nesse estudo, concentrado através da técnica TPP, mostrou-se complexo apresentando bandas distintas no seu perfil eletroforético.

- Ovinos infectados por *C. pseudotuberculosis* reconhecem principalmente uma banda de 32kDa, que possivelmente corresponde à exotoxina fosfolipase D, e outra de 70kDa, possivelmente uma proteína de choque térmico.

- O PCR multiplex (Pacheco *et. al.*, 2007) é capaz de identificar *C. pseudotuberculosis* com alta sensibilidade e especificidade, mostrando-se mais sensível do que o cultivo bacteriano.

- Na comparação com outras metodologias de diagnóstico, o ELISA desenvolvido com antígeno BHI demonstrou ser uma ferramenta segura de diagnóstico da infecção por *C. pseudotuberculosis.*

- Usando o ponto de corte de 204pg/ml de produção de IFN-y para caprinos e de 508pg/ml para ovinos, calculados pelo método de Frey, a sensibilidade do teste foi de 55.8% e 56%, enquanto a especificidade foi de 100% e 93%, respectivamente para cada espécie.

- Houve diferença estatística significativa em caprinos e ovinos na produção de IFN-y por animais infectados ou não com *C. pseudotuberculosis*.

imais negativos e animais em estado clínico da doença para caprinos e em ovinos para animais em estado clínico da linfadenite caseosa, se distinguindo de animais controle e subclínicos.

- A indução de IFN-y por antígenos secretados de *C. pseudotuberculosis* é um marcador de infectividade e de status clínico promissor em pequenos ruminantes.

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