UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

PERFIL DA EXPRESSÃO GÊNICA INDUZIDA PELA INFECÇÃO EXPERIMENTAL POR *Brucella ovis* EM TECIDOS REPRODUTIVOS E LINFÓIDES OVINOS

JOÃO MARCELO AZEVEDO DE PAULA ANTUNES

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Tese apresentada junto ao Programa de Pós-Graduação em Medicina Veterinária para obtenção do título de Doutor.

Orientadora: Profª. Drª. Jane Megid

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"A imaginação é mais importante que o conhecimento."

(Albert Einstein)

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ABC	ATP-Binding Cassete	NK	Células natural killers
BCVs	*Brucellas* containing vacuoles	qPCR	PCR em tempo real
BOPI-1	*B.ovis*-pathogenicity island 1	APC	Células apresentadoras de antígenos
BvrR/BvrS	Sistema de dois componentes BvrR/BvrS	IL	Interleucina
CR3	Complement receptor 3	RIH	Resposta imune humoral
DEGs	Differentially Expressed Genes	MIF	Macrophage migration inhibition factor
EEA1	Antígeno endossomal precoce 1	INF	Interferon
GTPases	Hidrolases de trifosfato de guanosina	PCR	Reação em cadeia pela polimerase
hmg	Gene da hemaglutinina	Ac	Anticorpos
kDa	kilodaltons	RI	Resposta imune
LAMP1	Proteínas associadas a membrana lisossomal 1	dpi	Dias pós infecção
LFA-1	Leucocyte function-associated antigen 1	RIC	Resposta imune celular
LPS	Lipopolissacarídeos	LBP	Lipopolysaccharide binding protein
MPR	Receptores de manose-6-fosfato	LTh1	Linfocito T helper 1
NODs	Nucleotide-binding oligomerization domain proteins	Ag	Antígenos
OMPs	Outer membrane proteins	DC	Células Dendríticas
ORFs	Open reading frames	SER	Sistema reticuloendotelial
PAMPs	Pathogen-associated molecular pattern	LTh2	Linfocito T helper 2
PRRs	Pattern recognition receptors	LT	Linfócitos T
RE	Retículo endoplasmático	RT-PCR	Reverse-transcriptase-PCR
T4SS ou *virB*	Sistema de secreção tipo-IV	TNF	Fator de necrose tumoral
TLRs	Tolll ike receptors		
wbo	Gene da glycosyltransferase	SFM	Sistema fagocítico mononuclear

Sumário

ANTUNES, J.M.A.P. **Perfil da expressão gênica induzida pela infecção experimental por *Brucella ovis* em tecidos reprodutivos e linfóides ovinos**. Botucatu, 2012. 101p. Tese (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista.

Resumo

Mundialmente a brucelose continua sendo um problema econômico e de saúde pública. A brucelose ovina é reconhecida como a mais importante causa de epididimite, sendo uma das principais causadoras de infertilidade em ovinos. Os mecanismos imunes envolvidos na defesa e eventualmente na persistência e manutenção do agente são desconhecidos. *Brucellas* spp. geram somente uma resposta inflamatória moderada e os estudos da patogenia da brucelose ovina são escassos. Recentes avanços demonstram que a resposta inflamatória induzida pelas *Brucellas* spp. representam provavelmente o resultado de uma tentativa de evasão e supressão da resposta imune hospedeira. Utilizando as tecnologias de RT-PCR em tempo real (q) e microarranjos este trabalho avaliou a expressão gênica da resposta imune e inflamatória na brucelose experimental ovina. O estudo apresenta a primeira análise do perfil de expressão gênica de citocinas através de RT-qPCR em tecidos reprodutivos e não reprodutivos de carneiros experimentalmente infectados com cepa de *B. ovis* PA até 240 dias pós infecção (dpi). O estudo também demonstra a primeira análise geral da expressão gênica por meio de microarranjos em tecidos reprodutivos e pool de linfonodos de carneiros experimentalmente infectados com cepa de *B. ovis* PA até 240 dpi. Neste trabalho demonstrou a persistência da *B. ovis* e o perfil inflamatório da resposta imune gerado pela infecção. Com este estudo espera-se aumentar o conhecimento da patogênese da *B. ovis* em carneiros infectados.

Palavras-chave: Brucelose; *Brucella ovis*; expressão gênica; RT-PCR; microarranjos.

ANTUNES, J.M.A.P. **Profile of gene expression induced by experimental infection with *Brucella ovis* in ovine reproductive and lymphoid tissues.** Botucatu, 2012. 101p. Tese (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista.

Abstract

Globally, brucellosis remains an economic problem and public health threat. The ovine brucellosis is recognized as the most important cause of epididymitis, one of the main causes of infertility in sheep. The mechanisms involved in immune defense and possibly the persistence and maintenance of the agent are unknown. *Brucellas* spp. generate only a moderate inflammatory response, and studies of the pathogenesis of ovine brucellosis are scarce. Recent developments have shown that *Brucella* spp. induces a moderate inflammatory response probably the result of an attempt to escape and to suppress the host immune response. Through real time (q) RT-PCR and microarrays the purpose this study evaluated the gene expression of immune and inflammatory response in experimental ovine brucellosis. The study demonstrated for the first time the gene expression profile of cytokines by RT-qPCR in reproductive and non reproductive tissues of rams experimentally infected with *B. ovis* PA strain up to 240 days post infection (dpi). The research also showed the first overall analysis of gene expression using microarrays in reproductive tissues and pool of lymph nodes of rams experimentally infected with *B. ovis* PA strain up to 240 dpi. The study demonstrated the persistence of *B. ovis* and the inflammatory profile of immune response occasioned due the infection. These results are expected to increase the knowledge of the pathogenesis of *B. ovis* in sheep.

Key words: Brucellosis; *Brucella ovis,* gene expression; RT-PCR; microarray.

I. INTRODUÇÃO

A brucelose é uma doença infectocontagiosa crônica comum a diversas espécies animais e é causada por cocobacilos Gram-negativos do gênero *Brucella*. Brucelas são bactérias patógenas dos mamíferos e são fortemente correlacionadas com outros parasitas intracelulares da classe α-Proteobacteria e gêneros *Orchrobactrum* (ETTEMA; ANDERSSON, 2009).

Mundialmente a brucelose é um problema econômico e de saúde pública com aproximadamente 500.000 casos por ano na espécie humana (TALESKI et al., 2002). As espécies conhecidas do gênero *Brucella* são *B. melitensis, B. abortus, B. suis, B. neotomae, B. ovis* e *B. canis* (LOPEZ et al., 1998), além de novas espécies em animais marinhos classificadas como *B. ceti* e *B. pinnipedialis* (FOSTER et al., 2002). Novas espécies de brucelas foram isoladas de animais selvagens (*B. microti*) (SCHOLZ et al., 2008), de implante de mama (SCHOLZ et al., 2010) e de pneumonia (TILLER et al., 2010), esta última denominada *B. inopinata*. As espécies clássicas mostram preferências por diferentes hospedeiros, mesmo exibindo 98% de homologia em seu DNA (FERRAZ, 1999). Seis das dez espécies de *Brucella* (*B. melitensis*, *B. abortus*, *B. suis*, *B. canis, B. ceti e B. pinnipedialis*) têm potencial zoonótico. A *B. melitensis* e a *B. suis* são as mais patogênicas, enquanto a *B. abortus* é considerada de patogenicidade moderada e a *B. canis* de baixa patogenicidade para o homem, enquanto que as brucelas marinhas possuem potencial de causar doença nos seres humanos (CLOECKAERT et al., 2001; PAPPAS, 2010). Não há relatos de infecção humana por *B. ovis* ou *B. neotomae* (CORBEL, 1997).

A brucelose ovina é reconhecida como a mais importante causa de epididimite ovina, sendo uma das principais causadoras de infertilidade nesta espécie (RIDLER; WEST, 2011). Embora encontrada em vários órgãos, *B. ovis* causa lesão específica em órgãos do trato reprodutivo masculino. Os fatores responsáveis pela persistência do agente nestes locais são desconhecidos, bem como os mecanismos imunes envolvidos na defesa e eventualmente na persistência e manutenção do agente (GIL-TURNES, 1998). Recentes avanços demonstram que *Brucella* spp. gera somente uma resposta inflamatória moderada que é provavelmente o resultado da tentativa de evasão da detecção

da resposta imune e supressão da resposta imune hospedeira (XAVIER et al., 2010). Atualmente não há estudos na patogenia e expressão de citocinas em brucelose ovina. Este estudo testou a hipótese de que diferentes níveis no padrão da expressão de citocinas podem influenciar na patogenia da enfermidade dependendo do órgão infectado, o que leva a *B. ovis* nos primeiros meses de infecção ficar restrita aos órgãos reprodutores (BIBERSTEIN et al., 1963). A resposta imune contra a *B. abortus* em bovinos é mediada predominantemente por uma resposta Th1 mediada por células, que envolve a ativação de linfócitos e secreção de IL-12 e INF-γ, que ativa macrófagos para o controle da infecção (ZHAN et al., 1996; RAJASHEKARA et al., 2006; MACEDO et al., 2008). Apesar do conhecimento acumulado nos últimos anos sobre a resposta imunológica na brucelose bovina, muito pouco é conhecido sobre a resposta imune contra *B. ovis.*

Segundo Lira e Megid (2009), de forma geral, inclusive no Brasil, são escassos os estudos da brucelose ovina, desta forma, neste trabalho objetivou-se determinar o perfil da expressão gênica através da avaliação de citocinas por PCR em tempo real (qPCR) e estudar a expressão gênica por microarranjo em tecidos reprodutivos e não reprodutivos de carneiros experimentalmente infectados com *B. ovis*.

II.REVISÃO DE LITERATURA

2.1 Epidemiologia

B. ovis foi descrita, inicialmente, na década de 50, na Nova Zelândia e na Austrália como um agente bacteriano que seria responsável por aborto e epididimite em ovinos; desde então estes microorganismos têm sido observados em vários países (BURGESS, 1982). A brucelose ovina foi relatada na maior parte das principais regiões produtoras de ovinos do mundo com exceção da Grã-Bretanha, sendo presente na Austrália, Nova Zelândia, América do Sul, América do Norte, Ásia Central, África do Sul e Europa (BURGESS, 1982). Em levantamento epidemiológico a ocorrência variou de 9,1 a 46,7% em áreas de criação intensiva de ovinos (SERGEANT, 1994). A soroprevalência em carneiros dentro de rebanhos positivos varia de 2,1 a 67% (SERGEANT, 1994; TORRES et al., 1997; ROBLES et al., 1998).

A brucelose ovina já foi identificada em diversos países, inclusive no Brasil (BURGESS, 1982; SANTOS et al., 2005a); foi diagnosticada nos estados de São Paulo (NOZAKI, 2002), Rio Grande do Norte (AZEVEDO et al., 2004), na Paraíba (CLEMENTINO et al., 2007), na Bahia (SILVA et al., 2009) e em Alagoas (PINHEIRO JÚNIOR et al., 2009), demonstrando grande disseminação pelo Brasil. No Rio Grande do Sul foi observada soroprevalência média de 13,4% (variando de 6,9 a 50%) em carneiros de rebanhos positivos, com maior positividade em animais mais velhos (MAGALHÃES NETO; GIL-TURNES, 1996). Em Minas Gerais, a análise de 833 amostras de soro, oriundas de 12 meso-regiões, resultou em 5,3% de ovinos reagentes para *B.ovis* e 29,4% de propriedades positivas (MARQUES, 2006).

2.2 Patogenia

B. ovis é um cocobacilo rugoso que produz doença clínica ou subclínica crônica em ovinos, que, além de ocasionar epididimite e subsequente diminuição de fertilidade em carneiros, pode ocasionar abortamentos ocasionais em ovelhas (SANTOS et al., 2005b; LÓPEZ et al., 2005). A principal forma de transmissão da brucelose ovina é a venérea, no momento em que um carneiro susceptível copula com uma ovelha (PAOLICCHI, 2001). Após a infecção por *B. ovis* nos animais susceptíveis, geralmente pelas mucosas,

ocorre lenta multiplicação do microrganismo devido à propriedade de resistir à destruição intrafagocitária. Ao final do segundo mês de infecção produz-se uma bacteremia e o agente passa a localizar-se em baço, rins e fígado, onde leva ao desenvolvimento de abscessos e reações inflamatórias crônicas. A bactéria se multiplica nos órgãos afetados e é eliminada à medida que as células infectadas são destruídas (GIL-TURNES, 1998).

B. ovis inicialmente se localiza nos linfonodos, dissemina-se por meio de bacteremia e finalmente coloniza o trato genital por volta dos 30 dias pós-infecção. Os fatores que definem o tropismo pelo trato genital ainda são desconhecidos (BIBERSTEIN et al., 1963).

2.3 Aderência, internalização e trânsito intracelular

A aderência de *Brucella* spp. ocorre por meio de receptores de moléculas contendo ácido siálico ou resíduos sulfatados, poucos minutos após sua adesão (CASTANEDA-ROLDAN et al., 2004). Martín-Martín et al. (2010) demonstraram que a penetração e internalização de *Brucella* spp. é dependente de dois componentes de lipídeos: os receptores de colesterol e o gangliosídeo GM1 classe A "*scavenger*", que limita a ativação de macrófagos, favorecendo a multiplicação intracelular e sua sobrevivência.

Outros mecanismos exatos da entrada de *Brucella* spp. são desconhecidos; entretanto, para *B. abortus,* há evidências que suportam a função da expressão pelas células do sistema imune inato dos *pattern recognition receptors* (PRRs) especialmente LFA-1 (*leucocyte function-associated antigen 1*), *complement receptor 3* (CR3), integrinas alpha-5, beta-1 e beta-3, receptores de manose-6-fosfato (MPR) e receptores Fc, que reconhecem os PAMPs (*pathogen-associated molecular pattern*) do patógeno mediando a aderência e internalização (CAMPBELL et al., 1994).

Após a aderência, o citoesqueleto de actina e microtúbulos participam da internalização de *Brucella* spp. No interior da célula ocorre ativação de hidrolases de trifosfato de guanosina (GTPases) da família Rho (Rho, Rac e Cdc42), proteínas envolvidas em diversos processos celulares que modulam a invasão por *Brucella* spp., como os componentes BvrS e BvrR, envolvidos na homeostase estrutural da proteínas externas de membrana (OMP3a e OMP3b) e que quando inativados são incapazes de ativar Cdc42 impedindo a invasão

celular por *B. abortus*. (GUZMÁN-VERRI et al., 2001). Uma falha na eliminação de *Brucella spp*. após sua internalização por macrófagos e consequente transporte para os linfonodos é responsável por sua disseminação para os órgãos e linfadenopatia regional (ENRIGHT et al., 1990). *Brucella* spp. opsonizadas ligam-se a células fagocitárias profissionais através do complemento e receptores Fc, enquanto em células não fagocitárias se ligam via lecitina e fibronectina (RITTING et al., 2001).

Após poucos minutos da invasão, *B. abortus* interage com compartimentos intracelulares, relacionados com a rede endossomal precoce, confirmada pela presença de marcadores como receptores de transferrina, pequenas GTP-*binding protein* rab5 e o antígeno endossomal precoce 1 (EEA1); (CHAVES-OLARTE et al., 2002). No trânsito dentro da célula do hospedeiro, o nicho intracelular das brucelas inclui os *Brucella containing vacuoles* (BCVs) precoces e os BCVs tardios acidificados, também chamados de brucellassomos. Após a internalização de *Brucella* spp., o BCV precoce será o compartimento que permitirá a sobrevivência da bactéria e o BCV tardio representa o compartimento da eliminação de *Brucella* spp. (RITTING et al., 2001). Os fagossomos ou BCVs precoces contendo *Brucella* marcados com Rab5 permitem limitada multiplicação de brucelas, mas não as eliminam (CHAVES-OLARTE et al., 2002). Os microrganismos do gênero *Brucella* se multiplicam em compartimentos retículo endoplasmático-*like*, como os BCVs, e lisam a célula hospedeira para sua disseminação (QIN et al., 2008).

Com o passar do tempo, continuando o trânsito intracelular, os BCVs tardios se apresentam marcados com proteínas associadas a membrana lisossomal 1 (LAMP 1) e marcadores do RE como calreticulina ou sec61β, porém desprovidos de marcadores como a catepsina D, que permitiriam sua eliminação e confirmando que as brucelas trafegam de autofagossomos para o RE, onde a multiplicação acontece (PIZARRO-CERDÁ et al., 1998). Mesmo com os BCVs estruturalmente marcados para ocorrer a fusão dos fagolisossomos, as brucelas conseguem inibi-la permitindo a sobrevivência intracelular nos macrófagos e alcançando o RE (NAROENI et al., 2001; CELLI et al., 2003). O *operon vir*B e a maquinaria do sistema de secreção tipo-IV (T4SS) regulam o tráfego intracelular, permitindo às brucelas alcançarem o RE (GORVEL; MORENO, 2002).

2.4 Fatores de virulência - *Brucella* spp.

Brucella spp. é um parasita intracelular-extracelular facultativo, que intercala suas vidas dentro e fora da célula do hospedeiro (GORVEL; MORENO, 2002). Os verdadeiros elementos de virulência de brucelas são os determinantes moleculares que permitem a ela invadir, resistir à morte e se replicar em células fagocitárias profissionais e não profissionais (GUZMÁN-VERRI et al., 2001). Brucelas lisas inibem a apoptose da célula hospedeira, enquanto brucelas rugosas como a *B. ovis* induzem a necrose do macrófago (PEI et al., 2006). Segundo SELEEM et al. (2008), as brucelas não possuem os genes clássicos de patogenicidade, e sim fatores de virulência que evitam a resposta imune e aumentam sua sobrevivência dentro da célula.

Bactérias do gênero *Brucella* possuem estratégias de evitar a sua detecção pelo sistema imune e promover uma infecção prolongada no interior das células, modificando a expressão dos genes de forma a sobreviver em vesículas acidificadas, alterando a apoptose de macrófagos, prevenindo a fusão do fagolisossomo e levando a uma menor ativação dos mecanismos de proteção e da inflamação (RAJASHEKARA et al., 2006).

A interferência no processo de fagocitose é o tempo necessário para *B. abortus* modificar a membrana plasmática da célula criando uma organela permissiva à sobrevivência e sua replicação intracelular (BELLAIRE et al., 2005). Muitos microrganismos do gênero *Brucella* são eliminados pelos fagolisossomos dos macrófagos, entretanto alguns evadem a fusão lisossomal e são capazes de se multiplicar amplamente durante o tráfego intracelular (GORVEL; MORENO, 2002). Uma vez dentro do fagossomo, sobrevivem à explosão respiratória e ao ambiente adverso por meio da interação do sistema de secreção tipo-IV (T4SS ou *vir*B) com o RE do hospedeiro (CELLI et al., 2005). O sistema T4SS codificado pelo gene *vir*B controla o tráfego intracelular e evita sua degradação inibindo a fusão fagossomo-lisossomo (COMERCI et al., 2001).

Nos hospedeiros, as brucelas residem em macrófagos, coordenando a expressão de múltiplos fatores de virulência para sua entrada e tráfego de forma a atingir seu nicho de replicação sem ativar fortemente o sistema imune

(RAMBOW-LARSEN et al., 2009). Diversos reguladores da expressão gênica foram identificados em *Brucella spp.*: *Quorum sensing, stringent response*, sistema regulatório de dois componentes e, mais recentemente descoberto, o *blue-light responsive* LOV-HK *protein,* que é responsável pela adaptação de brucelas a diferentes ambientes (RAMBOW-LARSEN et al., 2009).

O *Quorum sensing* em brucelas coordena a expressão de genes em diferentes condições ambientais, como a produção de fator de necrose tumoral-α (TNF-α). Devido a poucos nutrientes dentro do fagossomo, *Brucella* spp. depende de seu regulador de *stringent response* para sintetizar transportadores periplasmáticos como nutrientes (LAMONTAGNE et al., 2009).

O sistema regulatório de dois componentes BvrR-BvrS permite um ajuste individual da infecção e da expressão gênica em resposta a um estímulo ambiental e regula a fusão do fagolisossomo (RAMBOW-LARSEN et al., 2009).

2.5 Fatores de virulência - *Brucella ovis*

Cepas de brucela podem ser lisas ou rugosas dependendo da presença ou ausência da cadeia de polissacarídeo-O na molécula de lipopolissacarídeo (LPS). Cepas rugosas como *B. ovis* não possuem a cadeia-O, desta forma a cadeia de polissacarídeo-O não recobre outros antígenos da superfície como os das *outer membrane proteins* (OMPs), deixando-os mais expostos. O antígeno 29-kDa, um dos antígenos imunodominantes localizado no lipopolissacarídeo bacteriano, é composto de duas OMPs, a Omp25 e a Omp31 (VIZCAÍNO et al., 2001); a Omp25 está diretamente envolvida na penetração, replicação e sobrevivência de *B. ovis* no interior de células hospedeiras (CARO-HERNÁNDEZ et al., 2007; MARTÍN-MARTÍN et al., 2008).

Martín-Martín et al. (2010) demonstraram que a infecção por *B. ovis* em macrófagos utiliza receptores de superfície comuns às brucelas lisas: como os receptores de colesterol e o gangliosídeo GM1 classe A "*scavenger*".

TSOLIS et al. (2009), comparando o genoma de *B. ovis* com os genomas de *B. suis, B. melitensis* e *B. abortus,* relataram que no genoma de *B. ovis* faltavam genes que codificam proteínas. Neste mesmo trabalho também foi descrito que *B. ovis* possui 33 genes codificantes de proteínas únicas para esta espécie. Referem também que no cromossomo I de *B. ovis* não se

evidencia a região que contém o gene *wbo,* responsável por codificar *a* glicosiltransferase, essencial para a biossíntese do LPS. A falta deste gene contribui para o fenótipo rugoso da cepa. No cromossomo II, *B. ovis* contém uma ilha genômica única, de 26,5kb, específica para a espécie e chamada de *B.ovis-pathogenicity island* 1 (BOPI-1). Esta ilha genômica possui genes envolvidos na patogênese como open reading frames (ORFs), ATP-*Binding Cassete* (ABC), *transporter system ABC* (abcABCDE) e um provável gene de hemaglutinina (hmg). A presença de pseudogenes é relacionada com baixa virulência em humanos; o genoma de *B. ovis* possui 244 pseudogenes identificados (TSOLIS et al., 2009), sugerindo a associação com a menor patogenicidade desta espécie frente às demais do gênero.

O sistema de transporte ABC é requerido para *B. ovis* na sua sobrevivência intra-extracelular nas fases iniciais da infecção (SILVA et al., 2011). *B. ovis* codifica poucas proteínas do sistema de transporte ABC devido a sua quantidade de pseudogenes (TSOLIS et al., 2009), resultando em falha no transporte de certos nutrientes. Silva et al. (2011) ressaltam esta deficiência no transporte de nutrientes como responsável pela ausência de virulência completa de *B. ovis* em animais. Adicionalmente, os transportadores ABC de açúcares também possuem pseudogenes que reduzem a habilidade de *B. ovis* em utilizar a glucose e a galactose como fonte de energia, o que pode contribuir para o seu restrito tropismo de tecido e gama de hospedeiros (TSOLIS et al., 2009).

Com base na biotipagem, *B. ovis* é urease-negativa, entretanto em seu genoma possui dois agrupamentos de genes codificantes de urease com mutações, deleções e pseudogenes que sugerem sua inatividade ao teste de urease. Isso explica o fato de a infecção oral na brucelose ovina não ser importante, pois sendo urease negativa, não sobrevive à passagem pelo estômago (BANDARA et al., 2007).

Vários genes do metabolismo do açúcar que são funcionais nas espécies de *Brucella spp*. patogênicas ao homem são incompletos em *B. ovis* (TSOLIS et al., 2009). O eritritol é uma fonte de carbono na placenta para *B. abortus, B. suis, B. melitensis*, entretanto a brucelose ovina apresenta baixa incidência de abortos. Uma das possíveis explicações é o fato de que a *B. ovis* não oxida o eritritol e também seu crescimento não é inibido pela presença do

mesmo. Isto é decorrente da presença de *stop codon* em eryA e uma mutação em *eryD,* o que evita a entrada do eritritol na célula e impossibilita a sua utilização como fonte de carbono para o crescimento. Essas limitações também podem contribuir para a virulência limitada de *B. ovis* (TSOLIS et al., 2009).

A interação hospedeiro-patógeno pode ser definida em quatro tópicos: LPS, T4SS, proteínas autotransportadoras e OMPs. *B. ovis* possui um fenótipo rugoso de LPS, e o antígeno da cadeia-O que possui função crítica na patogenicidade de *B. abortus, B. suis e B. melitensis,* codificado pelo gene *wbo*A, é ausente no genoma da *B. ovis*. Esta diferença no LPS de *B. ovis* pode afetar a interação com *toll-like-receptors* (TLR) da resposta imune inata (TSOLIS et al., 2009).

O T4SS, que é codificado pelos genes *vir*B1-*vir*B12, é essencial para a virulência de cepas lisas (DELRUE et al., 2001). Os genes *vir*B1-*vir*B12 são intactos na *B. ovis,* sugerindo que este sistema seja funcional (TSOLIS et al., 2009).

É pressuposto que algumas proteínas estejam envolvidas no sistema de autotransporte, e cada espécie de brucela codificaria uma combinação proteínas (CHAIN et al., 2005), fato que sugere que nenhuma proteína seria essencial para a virulência, mas que diferentes combinações das mesmas podem contribuir no tropismo por tecidos e na gama de hospedeiros da *B. ovis*. A proteína autotransportadora OmaA é ausente na *B. ovis,* aspecto que também contribui para o limitado tropismo por tecidos e gama de hospedeiros (TSOLIS et al., 2009).

As OMPs são codificadas por dois componentes BvrR/BvrS, que são reguladores principais de muitas funções de virulência que se encontram intactas no genoma de *B. ovis* (GUZMAN-VERRI et al., 2001). Os dois genes omp2a e omp31 que codificam as OMPs possuem mutações em *B. ovis*, o que leva as OMPs de *B. ovis* a serem mais susceptíveis a peptídeos catiônicos (FREER et al., 1999) comprometendo a estabilidade da célula e tornando *B. ovis* menos capaz de sobreviver em ambientes desfavoráveis.

A biologia única de *B. ovis,* se comparada com as outras espécies patogênicas ao homem, em parte é resultado da degradação do seu genoma (TSOLIS et al., 2009). Foi comprovado, também, que a uréase, além de ter importância na infecção por via oral, também contribui para o estabelecimento

da infecção por via respiratória em suínos (BOSSE; MACCINES, 2000). Desta forma, possivelmente *B. ovis* não consegue se estabelecer no hospedeiro por via respiratória e nem por via oral, que são as duas principais portas de entrada de *Brucella* spp. patogênicas ao homem (TSOLIS et al., 2009).

2.6 Resposta imune

Bactérias do gênero *Brucella* induzem uma fraca resposta inflamatória, retardando uma resposta imune efetiva devido a um LPS pouco imunogênico que escapa da detecção pelos TLRs na resposta imune inata (LAPAQUE et al., 2006). A resposta inata ocorre imediatamente através das PRRs [TLRs, *nucleotide-binding oligomerization domain proteins* (NODs), *lipopolysaccharide binding protein* (LBP) e MPR] que se ligam aos PAMPs bacterianos (TIZARD, 2009). As brucelas produzem proteínas que inibem os sinais dependentes de TLRs que são responsáveis por maturação de células dendríticas (DC), secreção de citocinas/quimiocinas inflamatórias, afetando a resposta imune (RI) inata e adaptativa (SALCEDO et al., 2008; CIRL et al., 2008).

O reconhecimento dos PAMPs pelos PRRs resulta em opsonização, endocitose e fagocitose da bactéria e inicia a transcrição dos genes da resposta imune não específica. Os produtos destes genes incluem peptídeos antimicrobianos (GANZ; LEHRER, 1998) e citocinas inflamatórias (BARNES et al., 2002).

A ativação dos TLRs induz as células de defesa a sintetizar e secretar diversas citocinas, principalmente IL-1α e TNF-α, assim como IL-6, IL-12 e IL-18, desencadeando-se inflamação e resposta de fase aguda (TIZARD, 2009). Entretanto, a mesma ativação pode ocasionar inibição da RI inata, secreção de citocinas, maturação de fagócitos, processamento de antígenos e apresentação de antígenos a linfócitos T (LT) (CIRL et al., 2008). Também é relatado que a *B. suis* induz o bloqueio da produção de TNF-α (BILLARD et al., 2007).

No estudo sobre expressão gênica na brucelose experimental ovina, utilizando a cepa de *B. ovis* PA, GALINDO et al. (2009), demonstraram que até 60 dias pós-infecção (dpi) em leucócitos existe um aumento de expressão (*upregulation*) de genes envolvidos com a ativação da resposta inflamatória e fagocitose e uma diminuição de expressão (*downregulation)* dos genes

envolvidos com os mecanismos de proteção favorecendo a cronicidade do processo e mantendo um perfil de resposta imune celular (RIC) com LT helper 1(LTh1).

A natureza intracelular do agente estimula uma resposta imune celular (RIC) do tipo LTh1 (VEMULAPALLI et al., 2000). Linfócitos T são diferenciados em duas classes: Th1 e LT helper 2 (Th2). A subclasse Th1 secreta principalmente IL-2, INF-γ, TNF-α, que participam da RIC; as da subclasse Th2 secretam IL-4, IL-5, IL-6 e IL-10, promovendo a resposta imune humoral (RIH) (SALAS-TÉLLEZ et al., 2005). Estudos demonstraram que ambas, RIC e RIH, participam na proteção contra a brucelose. A localização intracelular requer uma estimulação da RIC (Th1); entretanto, pesquisas em camundongos caracterizaram que a imunidade protetora contra brucelose é devida a uma combinação entre anticorpos (Ac) e RIC (FICHT; ADAMS, 2009).

O perfil da expressão gênica da RI da brucelose ovina em tecidos reprodutivos e não reprodutivos ainda não foi descrito. Considerando a necessidade de estudos na patogenia da *B. ovis*, o presente estudo objetivou determinar o perfil da expressão gênica em carneiros experimentalmente infectados com *B. ovis* em tecidos reprodutivos e não reprodutivos através da avaliação de citocinas por PCR em tempo real (qPCR) e avaliação de genes da resposta imune por microarranjos.

III.TRABALHO CIENTÍFICO 1[1]

Rough virulent strain of *Brucella ovis* induces pro- and anti-inflammatory cytokines in reproductive tissues in experimentally-infected rams

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ABSTRACT

Cytokine expression profiles were studied for eight months in rams after being experimentally infected with the rough virulent strain of *Brucella ovis* (R-*B.ovis*). The mRNA expression levels of IL-1α, IL-1β, IL-6, IL-10, IL-12, INF-γ and TNF-α cytokines were quantified by real-time quantitative RT-PCR (qRT-PCR) in reproductive tissues (epididymus, testicles, ampolae, vesicular glands, bulbourethral glands), and non-reproductive (liver, spleen, kidneys) tissues at 30, 60, 120, 240 days post infection (dpi). During the acute phase of infection at 30 dpi the host immune response was most notable demonstrating an upregulation of several cytokines in reproductive tissues, including the epididymus (IL-6, IL-1β, IL-1α), testicles (INF-γ, IL-12), bulbourethral glands (IL-6, TNF-α) and ampolae (INF-γ, IL-10, IL-1β, IL-1α). During the development of infection, cytokine gene expression levels decreased, providing evidence of immune evasion that favored persistence of chronic R-*B. ovis* infection. During the chronic phase of R-*B. ovis* infection (120 and 240 dpi), mRNA cytokine production was downregulated in the epididymus (IL-1β, IL-1α), testicles (INF-γ, IL-12), and ampolae (INF-γ, IL-10, IL-1β, IL-1α), with the exception of the bulbourethral glands (IL-6 and TNF-α) and epididymus, (IL-6); in these tissues R-*B.ovis* infection resulted in upregulation of the proinflamatory cytokine IL-6. Herein, we report cytokine expression profiles in tissues of rams experimentally infected with the rough strain of *B. ovis*, which are associated with bacterial persistence and macrophage activation.

Keywords: *Brucella ovis*, brucellosis, cytokines, rough strain, ram, gene expression.

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1. Introduction

Brucellosis is one of the most important infectious diseases that causes reproductive disorders in domestic animals (Megid et al., 2010). *Brucella* spp. replicates and persists within macrophages in various tissues and induces a moderate inflammatory response (Moreno and Gorvel, 2004; Xavier et al., 2010), while avoiding the immune response in favor of bacterial persistence (Seleem et al., 2008). *Brucella ovis* infection in sheep causes ovine brucellosis or contagious epididymitis (Rajashekara et al., 2006). *B. ovis* has a predilection for replication in the genital tract of rams, and infection in these tissues causes epididymitis and orchitis (Tsolis et al., 2009; Ridler and West, 2011). The mechanism which favors the *B. ovis* tropism for genital tract has not been reported (Tsolis et al., 2009). During the first months of infection *B. ovis* persists mainly in reproductive tissues where the bacteria is phagocytized by resident macrophages and dendritic cells, after which the infection becomes disseminated in reticuloendothelial and non-reproductive tissues (Birberstein et al., 1963; Xavier et al., 2010). In rams *B. ovis* induces the upregulation of pro-inflammatory genes in leukocytes (Galindo et al., 2009). In this study, expression of cytokine genes in reproductive and non-reproductive tissues of rams over the duration of eight months after being experimentally-infected with the rough (R) virulent strain of *B. ovis* was studied using real time qRT-PCR.

2. Materials and methods

2.1 Infection of sheep

Twenty-four, one year-old Santa Inês rams, previously determined to be free of brucellosis, visna-maedi, toxoplasmosis and neosporosis, were used for this study. The rams were randomly assigned to one of two treatment groups. One group of 12 rams was kept in an isolated pen and experimentally-infected with the PA strain (ATCC25840) virulent rough (R) of (R-*B. ovis)*(Blasco et al., 1990) provided by Dr. Renato L. Santos (Universidade Federal de Minas Gerais, UFMG, Belo Horizonte, Minas Gerais, Brazil). Experiments were conducted with the approval of Ethical Committee of UNESP (Ethics committee protocol 69/2008). The R-*B. ovis* strain was cultured on blood agar plates and incubated for 48h at 37^{o}C in 10% CO_2. Bacteria were then washed with 10mM phosphate buffered saline (PBS; pH 6.8) and the concentration was adjusted using a spectrophotometer (550nm). The rams were each infected with 2.2 x 10^9 *B. ovis* CFU doses (30 μl administered in the conjunctiva and 30 μl injected intrapreputially, total 60 μl). The uninfected, control rams were injected in the same manner with sterile distilled water. At each of four time periods (30, 60, 120, and 240 days post infection (dpi) three infected and three non-infected were euthanized and the reproductive (epididymus, testicles, ampolae, vesicular glands, bulbourethral glands), and non-reproductive (liver, spleen, kidneys) tissues were collected and tested by PCR to confirm R-*B. ovis* infection (Fig.1) (Manterolla et al., 2003). R-*B. ovis* infection in rams was also confirmed at 15 dpi by serology using the agar gel immunodiffusion (AGID) test (Instituto de Pesquisas Veterinárias Desidério Finamor – IPVDF, Eldorado do Sul/RS, Brazil).

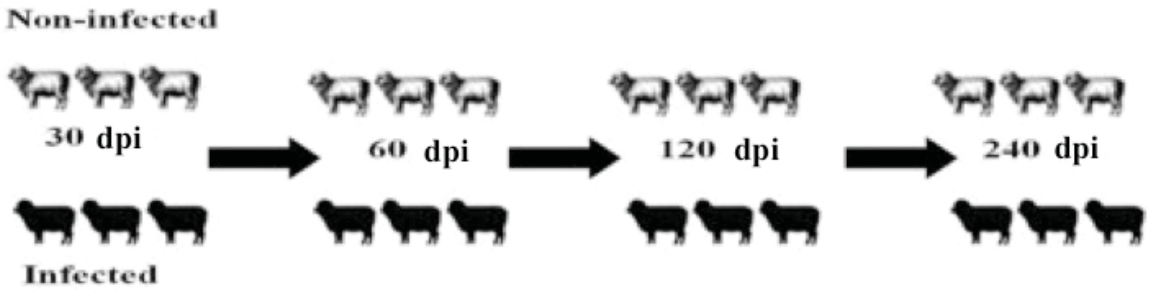


Fig.1. Experimental design. Differential expression of genes from infected rams was compared with control by qRT-PCR analysis during the course of infection. Six rams were sampled at each time point of infection; dpi (days post infection).

2.2 qRT-PCR

The study was done in accordance with the guidelines of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) (Bustin et al., 2009). Total RNA was extracted using the Invisorb® Spin Tissue RNA Mini Kit (Invitek Gmbh, Germany) according to the manufacturer's instructions, and the RNA was quantified by absorbance at 260 nm. Reverse transcription was performed using 1μg DNase-treated total RNA in the presence of 1 mmol/L oligo(dT) primer, 4 U Omniscript RTase (Qiagen, Mississauga, ON, Canada), 0.25 mmol/ L dideoxynucleotide triphosphate (dNTP) mix and 19.33 U RNase Inhibitor (GE Healthcare, Baie D'Urfé, QC, Canada) in a volume of 20 μL at 37^{o}C for 1 h, followed by incubation at 93^{o}C for 5 min. qPCR was performed using the 7300 Real-Time PCR system (Applied Biosystems, Streetsville, ON, Canada) with the Power SYBR Green PCR Master Mix. The ovine-specific cytokine primers for target genes (IL-1α, IL-1β, IL-6, IL-10, IL-12, INF-γ and TNF-α) were designed and recorded at the portal for qPCR primers, RTPrimerDB (http://www.rtprimerdb.org). Common thermal cycling parameters (3 min at 95^{o}C, 40 cycles of 15 sec at 95^{o}C, 30 sec at 59^{o}C, and 30 sec at 72^{o}C) were used to amplify each transcript. The melting-curve analyses were

performed to verify product identity. Samples were run in duplicate and were analyzed in comparison with *Ovis aries* beta-actin gene validated previously in gene expression (GE) experiments (Galindo et al., 2009). The data were normalized using the ΔΔCt method with correction for amplification efficiency (Pfaffl, 2001).

2.3 Statistical analysis

In order to compare the levels of mRNA cytokines as a response to treatment time (control vs infected; 30, 60, 120 and 240 dpi) and their interaction, generalized mixed linear models were designed separately for each cytokine and organ. Each individual was included as a random factor with a normal distribution and an identity link. The significance was set at a P-value ≤ 0.05. All analyses were done using SAS statistical software (SAS Institute Inc. Cary, North Carolina, USA).

3. Results and discussion

The generalized mixed linear models of gene expression in R-*B. ovis* infected rams were done separately for each cytokine and organ, and the results are listed in Table 1. The interaction between time and treatment in the epididymus was significant for IL-6 and IL-1α and in the testicles for IFN-γ and IL-12. The pattern was similar in infected individuals at the beginning of the infection, but with higher gene expression values that decreased over time.

Table 1- Results of the generalized mixed linear models on gene expression in R-*B. ovis*-infected rams, which were carried out separately for each cytokine and organ.

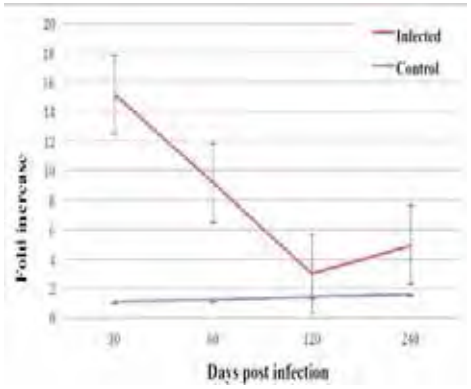
Dependent variable	Epididymus	Testicles	Vesicular glands	Bulbourethral glands	Ampolae	Liver	Spleen	Kidneys
IFN-γ	n.s.	T ** (*F*= 6.1) Ti X T** (*F*=7.0)	n.s.	n.s.	T**(*F*=10.6)	n.s.	n.s.	n.s.
IL-6	Ti***(*F*=9.2) T***(*F*=38.1) Ti X T***(*F*=10.6)	n.s.	n.s.	T*(*F*=8.1)	n.s.	n.s.	n.s.	n.s.
IL-10	n.s.	n.s.	n.s.	n.s.	T*(*F*=6.0)	n.s.	n.s.	n.s.
IL-12	n.s.	Ti***(*F*=13.7) T**(*F*=15.7) Ti X T***(*F*=13.0)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
IL-1β	Ti*(*F*=4.2)	n.s.	n.s.	n.s.	T*(*F*=5.8)	n.s.	n.s.	n.s.
IL-1α	Ti X T*(*F*=3.2)	n.s.	n.s.	n.s.	T*(*F*=6.7)	n.s.	n.s.	n.s.
TNF-α	n.s.	n.s.	n.s.	T*(*F*=6.7)		n.s.	n.s.	n.s.

*= <0.05; ** = <0.01; *** = <0.001; Ti= Time; T= treatment; Ti X T= Time X Treatment; *F*= F-valor; n.s.= non-significant

Treatment alone, independently of time, was significant in epididymus for IL-1β and IL-6, testicles for INF- γ and IL-12, bulbourethral glands for IL-6 and TNF-α in, and ampolae for IFN-γ, IL-10, IL-1β, and IL-1α, and gene expression was always higher in infected individuals. Time alone, independently of treatment, was significant in epididymis for IL-6 and IL-1β and in testicles for IL-12. Significant results were not obtained in vesicular glands and the non-reproductive tissues, liver, spleen and kidneys. All rams from the infected group were serologically positive after 15 dpi, and reproductive tissues from the infected sheep were determined to positive for *Brucella* by PCR after necropsy.

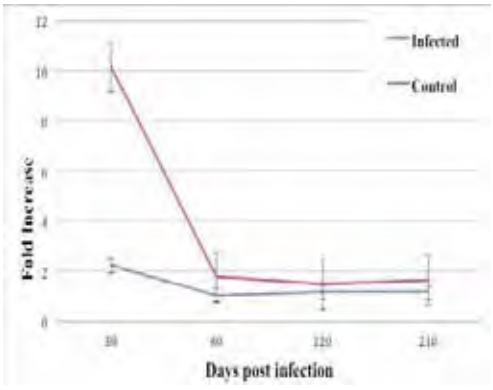
The use of qPCR for quantification of mRNA cytokines in sheep was previously reported (Smeed et al., 2007). Upregulation of pro-inflammatory and non-inflammatory cytokines in reproductive organs provided evidence of an immune response (Fig.2).

Clinical signs of ovine brucellosis, including epididymitis and orchitis, were observed as described previously by Megid et al. (2010). While previous studies demonstrated the upregulation of pro-inflammatory genes as IL-1α in infected rams (Galindo et al., 2009), and the presence of the bacteria in reproductive tissues from infected rams did not result in cytokine downregulation at 30 dpi. While upregulation of the cytokines in reproductive tissues may initially be directed toward elimination of bacteria, immunity against brucellosis also involves Th1 and Th2-type responses (Leal-Hernandez et al., 2009). Rafiei et al. (2006) reported a switching of cytokine Th1-type at disease onset followed by Th2-type during the progression of infection in human brucellosis. Galindo et al. (2009) reported at 60 dpi a Th1 profile and a suppression of Th2-type response. In the present study at late phases of infection (120 and 240 dpi) a remarkable upregulation of cytokines occurred in bulbourethral glands (Fig. 2 f and Fig. 2 g) and traces of Th1-type cytokines were found in other reproductive tissues (Fig. 2 b-e and Fig. 2 h-k) however, the expression of IL-10 a Th2-type cytokine was visualized in ampolaes (Fig. 2 i). At late phase of infection, the cytokine profile is associated with the persistence of *B. ovis* in rams (Fig. 2).



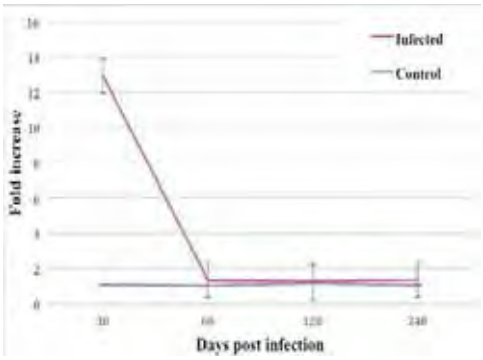
a

Epididymus IL-6



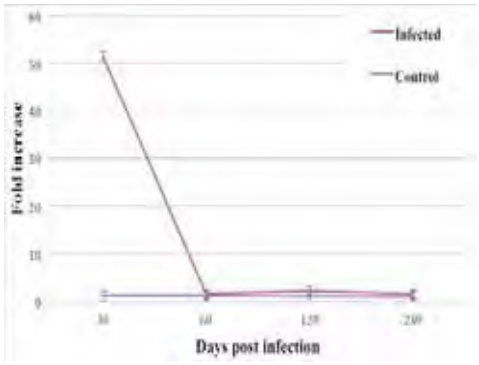
b

Epididiymus IL-1β



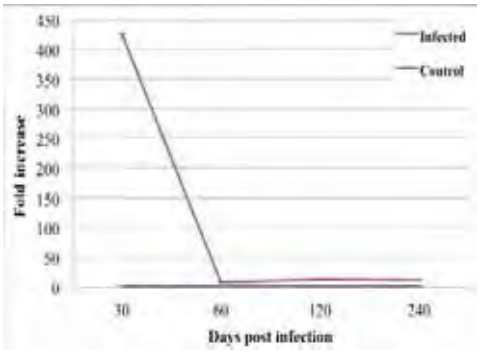
c

Epididymus IL-1α



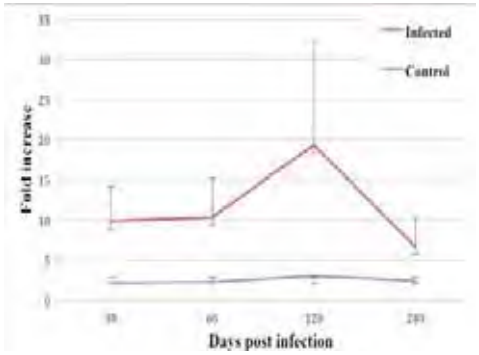
d

Testicles INF-γ



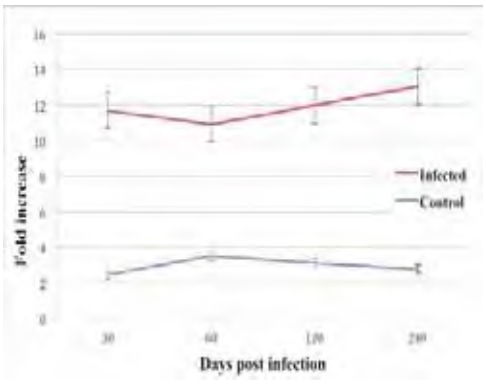
e

Testicles IL-12



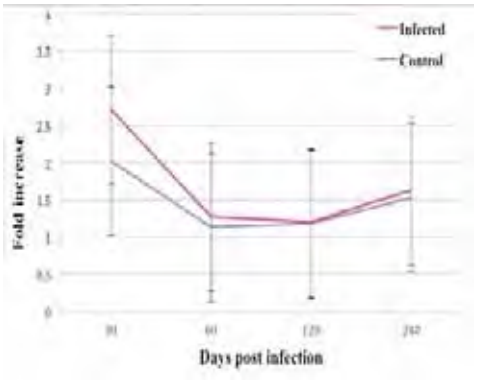
f

Bulbourethral glands IL-6



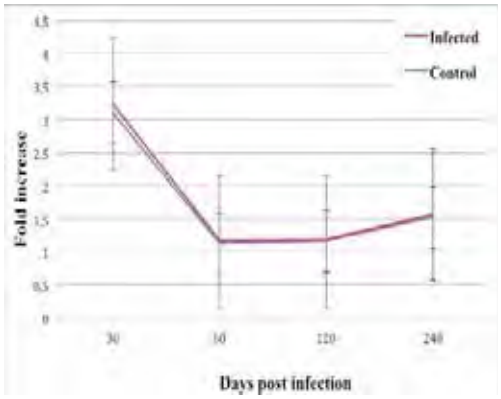
g

Bulbourethral glands TNF-α



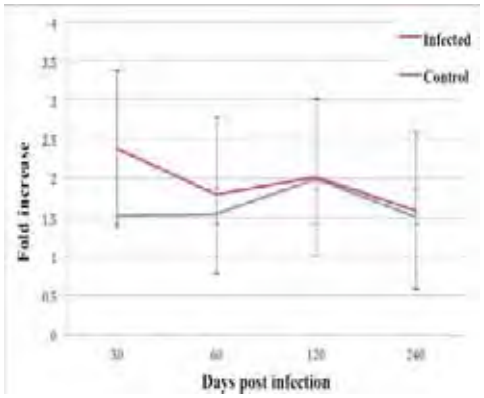
h

Ampolae INF-γ



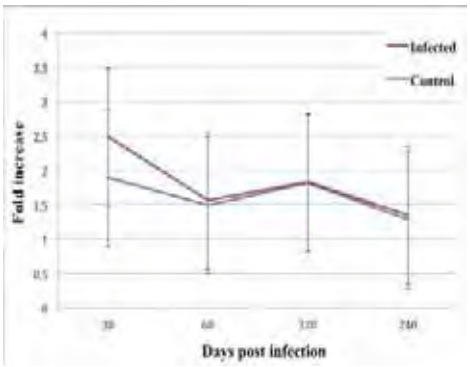
i

Ampolae IL-10



j

Ampolae IL-1β



k

Ampolae IL-1α

Fig.2. Expression of selected genes in rams with R-*B. ovis* experimentally infected. The expression of the mRNA from selected genes was quantified by qRT-PCR in epididymus: IL-6 (a), IL-1β (b), IL-1α (c); testicles: INF-γ (d), IL-12 (e); bulbourethral glands: IL-6 (f), TNF-α (g); ampolae: INF-γ (h), IL-10 (i), IL-1β (j), and IL-1α (k). Relative mRNA was measured by qPCR RT-PCR, and data expressed as ΔΔCt.

The relative mRNA expression of pro-inflammatory and non-inflammatory cytokines in the reproductive tissues in the current study demonstrated activation of both innate and adaptive immune responses. The upregulation of cytokines was not observed in liver, spleen and kidneys, confirming that R-*B. ovis* has a tropism for reproductive tissues (Ridler and West, 2011). Vesicular glands have been reported to be a site of predilection for *B. ovis* replication, however in this study cytokine expression on this tissue was not significant (Biberstein et al., 1964). Most of the cytokine levels decreased during the course of infection in reproductive tissues (Fig.2), suggesting that pathogenic factors may allow *B. ovis* to evade the host immune response (Tsolis et al., 2009). The presence of IL-6, in epididymus and bulbourethral glands, represents macrophage and acquired immune response activations (Salas-Téllez et al., 2005). The presence of IL-12 in testicles, a growth and maturation factor that affects the innate and adaptive immune response, suggests cell-mediated cytotoxicity, a response to bacterial infection (Rose et al., 2000). The decreasing of cytokines levels in reproductive tissues was expected because of the inhibition of antigen presentation against *Brucella* spp. (Barrionuevo et al., 2008; Martirosyan et al., 2011). According to Galindo et al. (2009), *B. ovis* induces downregulation of genes in order to suppress the host response. In ampolae a slight upregulation of cytokines was observed, including the anti-inflammatory cytokine IL-10. Golding et al. (2001) reported that IL-10 controls intracellular growth of *Brucella spp*. When the infection had progressed to 120 dpi in reproductive tissues (epididymus, Fig. 2 b-c; testicles, Fig. 2 d-e; and ampolae, Fig. 2 h-k), with exception of bulbourethral glands (Fig. 2 f-g), and epididymus (Fig. 2 a), cytokines levels decreased. The reduction of cytokines levels suggested the establishment of chronic *B. ovis* infection. The upregulation of mRNA genes at 120 dpi (IL-6, TNF-α, Fig. 2 a, f, g) may be an antibacterial host mechanism directed toward bacterial elimination (Eskra et al.,

2003). IL-6/TNF-α (bulbourethral glands), and IL-6 (epididymus) cytokines were increased at 120 dpi. IL-6 is stimulates by IL-1α, TNF-α, secretion and links the innate and adaptive arms of the immune system (Rose et al., 2000). The increase of pro-inflammatory IL-6 cytokines levels in the epididymus and bulbourethral glands during chronic late phase could be a mechanism used by the host to eliminate *B. ovis* infection. Barrionuevo et al. (2008) recently demonstrated that infection with *Brucella spp*. downregulates expression of antigen presentation, a mechanism mediated by IL-6, promoting chronicity of brucellosis. The presence of TNF-α was reported for controlling the growth of *Brucella* (Ko and Spliter, 2003; Barquero-Calvo et al., 2007), and as reported by Gorvel and Moreno (2002) the inflammatory cytokines profile inhibits the growth of *Brucella spp*. At the chronic phase of infection (240 dpi) cytokine levels appeared to be suppressed by R-*B. ovis* infection, and only TNF-α continued to increase in bulbourethral glands (Fig. 2 g).

4. Conclusions

In this study, cytokine gene expression profiles were determined by qPCR of selected tissues from rams infected with R-*B. ovis* as compared with tissues from uninfected controls. During the acute phase of infection (30 dpi) upregulation of cytokines in reproductive tissues (epididymus, IL-6, IL-1β, IL1-α; testicles, INF-γ, IL-12; bulbourethral glands, IL-6, TNF-α; and ampolae, INF-γ, IL-10, IL-1β, IL-1α) provided evidence of a host immune response. The decreased expression of IL1-α, IL-1β and INF-γ in the epididymus, testicles, and ampolae during R-*B. ovis* infection suggested inhibition of an immune response. The lower levels of cytokines in reproductive tissues after 30 dpi (with the exception of IL-6 and TNF-α for bulbourethral glands, and IL-6 for epididymus) suggested that a mechanism of evasion of the immune response in

favor of the persistence of chronic R-*B. ovis* infection. During the chronic phase of R-*B. ovis* infection (120 and 240 dpi), downregulation of cytokines (except in bulbourethral glands with IL-6 and TNF-α; IL-6 for epididymus) occurred in the epididymus (IL-1β, IL-1α), testicles (INF-γ, IL-12) and ampolae (IL-10, INF-γ, IL-1β, IL-1α) indicating a cytokine profile that not induces macrophage activation and stimulates the persistence of *B. ovis* in rams. The results of this study have expanded our understanding of the pathogenesis of R- *B. ovis* infection in ram reproductive tissues.

Conflict of interest statement

All authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence their work.

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IV.TRABALHO CIENTÍFICO 2[2]

Microarray analysis of gene expression in rams experimentally-infected with the rough virulent strain of *Brucella ovis*

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ABSTRACT

Microarray analysis of gene expression profiles of total mRNA was studied in rams experimentally-infected with the rough virulent strain of *Brucella ovis* (R-*B. ovis*). The microarray analysis of each tissue was done at three collection times: acute infection (60 days post infection [dpi]), chronic infection phase 1 (120 dpi), and chronic infection phase 2 (240 dpi), and the tissues studied included the reproductive organs (epididymus, testicles, ampolae, vesicular glands, bulbourethral glands) and a pool of lymph nodes (inguinal and scrotal). The gene expression profiles associated to R-*B. ovis* infection were determined using the Affymetrix Bovine Genome Array and expression levels of infected and non infected rams (control group, 0 dpi) were compared. Of the 23,000 genes analyzed in the microarrays, 139 during acute phase of infection, 930 during the chronic phase I and 744 from the chronic phase II were identified as Differentially Expressed Genes (DEGs). Forty-four DEGs (30 known genes and 14 unknown genes) were expressed during the three phases of infection. The biological functions of these genes included immune cell trafficking, immunological disease, infectious disease, inflammatory disease, inflammatory response and cellular movement and these differences were significant at the three phases of infection. The results reported herein represent the first microarray analysis performed on tissues of rams infected with R- *B. ovis*.

Keywords: *Brucella ovis*, brucellosis, microarray, sheep, rams, gene expression.

1. Introduction

Brucella ovis is the causative agent of ovine brucellosis or contagious epididymitis, a disease mainly characterized by infertility in rams (Megid et al., 2010). *Brucella spp*. replicate and persist within macrophpages in host tissues, including male and female

reproductive tissues (Moreno and Gorvel, 2004). Infection of these tissues with *B. ovis* results in a moderate inflammatory response, and virulence factors promote evasion of the immune response persistent infection (Seleem et al., 2008; Xavier et al., 2010). Previous microarrays studies of gene expression patterns were done using buffy coat cells until 60 dpi in rams experimentally-infected with *B. ovis* (Galindo et al., 2009). The use of heterologous array hybridization (screening for gene expression in one species using a microarray developed for another species) has been previously done (Galindo et al., 2009; Pariset et al., 2009). In this study, characterization of gene expression using total mRNA was done by microarray hybridization and real-time qRT-PCR of multiple tissues collected from rams experimentally-infected with R-*B. ovis* over of eight months dpi.

2. Results

All rams from infected groups were seropositive after 15 dpi, and tissues samples from each infected group collected after euthanasia were PCR positive.

2.1 Acute phase of infection (60 dpc)

After microarray analysis of the 23,000 genes at 60 dpi, 139 were common DEGs in reproductive tissues and pool of lymph nodes, but only 119 genes had a fold change ≥ 1.3. From the 139 DEGs in this group, 119 were upregulated (83 known and 36 unknown genes) and 20 were downregulated (16 known and 4 unknown genes). The IPA tool (Ingenuity Pathway Analysis, http://www.ingenuity.com) was subsequently used to characterize the 139 DEGs for detection of biological functions and main canonicals pathways (Table 2).

Table 2. Canonical pathways associated with the DEGs from 60 dpi.

Ingenuity Canonical Pathways[a]	**p-value**[b]
IL-3 Signaling	1.98E00
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	1.86E00
p53 (tumor protein 53) Signaling	1.82E00
Role of NFAT (nuclear factor of activated T cells) in Regulation of the Immune Response	1.8E00
T Cell Receptor Signaling	1.73E00
CD28 Signaling in T Helper Cells	1.52E00
EGF (Epidermal growth factor)Signaling	1.42E00
IL-2 Signaling	1.29E00
GM-CSF (Granulocyte-macrophage colony-stimulating factor) Signaling	1.21E00
CD40 Signaling	1.19E00
Macropinocytosis Signaling	1.15E00
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	1.04E00
CTLA4 (Cytotoxic T-Lymphocyte Antigen 4) Signaling in Cytotoxic T Lymphocytes	1.03E00
SAPK/JNK (stress-activated protein kinase/c-Jun NH2-terminal kinase) Signaling	1.02E00
IL-12 Signaling and Production in Macrophages	8.96E-01
IGF-1 (insulin-like growth factors) Signaling	8.78E-01
TNFR2 (Tumor necrosis facror recptor 2) Signaling	7.65E-01
IL-9 Signaling	7.32E-01
MIF (Macrophage migration inhibition factor) Regulation of Innate Immunity	7.02E-01
B Cell Receptor Signaling	6.89E-01
B Cell Activating Factor Signaling	6.62E-01
IL-17A Signaling in Fibroblasts	6.62E-01
Clathrin-mediated Endocytosis Signaling	6.3E-01
Endothelin-1 Signaling	6.24E-01
TNFR1(Tumor necrosis facror recptor 1) Signaling	6.14E-01
CXCR4 (C-X-C chemokine receptor type 4) Signaling	6.13E-01
CD27 Signaling in Lymphocytes	6.03E-01
Toll-like Receptor Signaling	5.82E-01
LPS/IL-1 Mediated Inhibition of RXR (Retinoid X receptor beta (RXR-beta) Function	5.72E-01
Acute Phase Response Signaling	5.53E-01
IL-8 Signaling	5.43E-01
Leukocyte Extravasation Signaling	5.39E-01
Molecular Mechanisms of Cancer	5.37E-01
Role of JAK1 (Janus kinase 1)and JAK3 (Janus kinase 3) in γc Cytokine Signaling	5.27E-01
IL-4 Signaling	5.19E-01
IL-17A Signaling in Airway Cells	5.11E-01

JAK/Stat (Janus kinase/ Signal Transducer and Activator of Transcription) Signaling	4.95E-01
Chemokine Signaling	4.95E-01
IL-10 Signaling	4.87E-01
IL-15 Signaling	4.87E-01
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	4.66E-01
CCR5 (C-C chemokine receptor type 5)Signaling in Macrophages	4.53E-01
IL-17 Signaling	4.46E-01
TGF-β (Transforming growth factor beta) Signaling	4.22E-01
Natural Killer Cell Signaling	3.64E-01
IL-1 Signaling	3.55E-01
VEGF (Vascular endothelial growth factor) Signaling	3.5E-01
Virus Entry via Endocytic Pathways	3.46E-01
IL-6 Signaling	3.46E-01
Fc Epsilon RI Signaling	3.37E-01
CCR3 C-C chemokine receptor type 3)Signaling in Eosinophils	3.21E-01
Cdc42 (Cell division control protein 42) Signaling	2.99E-01
Dendritic Cell Maturation	2.6E-01
NF-κB ((nuclear factor kappa-light-chain-enhancer of activated B cells) Signaling	2E-01

[a] Functional annotation analysis of the differentially expressed genes (DEGs) with the IPA (Ingenuity Pathway Analysis, http://www.ingenuity.com) tools.

[b] P value determined from the average log2 ratio using Bioconductor (http://www.bioconductor.org).

After the clustering analysis, tissues were grouped according to their disease condition (Figure 2). By use of the IPA software cellular commitment after infection was demonstrated by the top functions: infectious diseases, immunological disease, inflammatory disease, protein degradation, protein synthesis, cancer, gene expression, cell cycle, nucleic acid metabolism, small molecule biochemistry, cell signaling, DNA replication, recombination, and repair, and cell-to-cell signaling and interaction. The IPA tools also identified DEGs significantly associated with biological functions: immunological disease, inflammatory diseases, infectious diseases, tumor morphology

and reproductive system disease. The top functions displayed by IPA tool suggested that upregulated genes are ones involved in the immune response against R-*B. ovis* infection in rams (Figure 3).

2.2 Chronic phase I of infection (120 dpc)

After microarray analysis of the 23,000 genes in tissues collected at chronic phase I of infection, 930 genes were found to be DEGs, 800 of these genes displayed a fold change expression ≥1.2 x. From the total of 930 DEGs, 800 genes were upregulated (613 known and 187 unknown genes), and 130 were downregulated (88 known and 42 unknown genes). Subsequently, the 930 DEGs were characterized using the IPA tool detection of possible biological functions and main canonicals pathways (Table 3). At 120 dpi, all canonical pathways were demonstrated. During acute phase of infection were also observed, as well as new pathways associated with the DEGs (Table 3) thus providing evidence of innate and adaptive immune responses. IPA tool demonstrated that the DEGs were mainly associated with genetic disorders such as cell death, immune cell trafficking, gene expression, inflammatory response, antigen presentation, and inflammatory/immunological/infectious diseases, and cancer.

2.3 Chronic phase II of infection (240 dpc)

From the 23.000 genes analyzed by microarray during the chronic phase II of infection at 240 dpi, 744 were identified as DEGs. Of these DEGS, 658 genes displayed a fold change ≥1.2, and 658 were upregulated genes (575 known and 83 unknown genes), while 83 were downregulated (63 known and 23 unknown genes). The IPA tool was then used to characterize the 744 DEGs in order to detect biological significance. The IPA tool identified several pathways and functions: IL-8 signaling pathway, clathrin-mediated endocytosis signaling, leukocyte extravasation signaling, antigen presentation

pathway, Fcγ receptor pathway, dendritic cell maturation, coagulation system, endocytic pathways, LPS/IL-1, JAK1, JAK2 and TYK2 related to interferon signaling, NF-κB activation, IL-22 signaling and interferon signaling pathway. An association between DEGs and top functions networks was also performed using the IPA, which demonstrated many functions including cell morphology, gene expression, cellular movement, cellular assembly and organization, immunological disease, cell-to-cell signaling and interaction, cancer, infectious disease, reproductive system disease, inflammatory response, cell death, DNA replication, recombination, and repair.

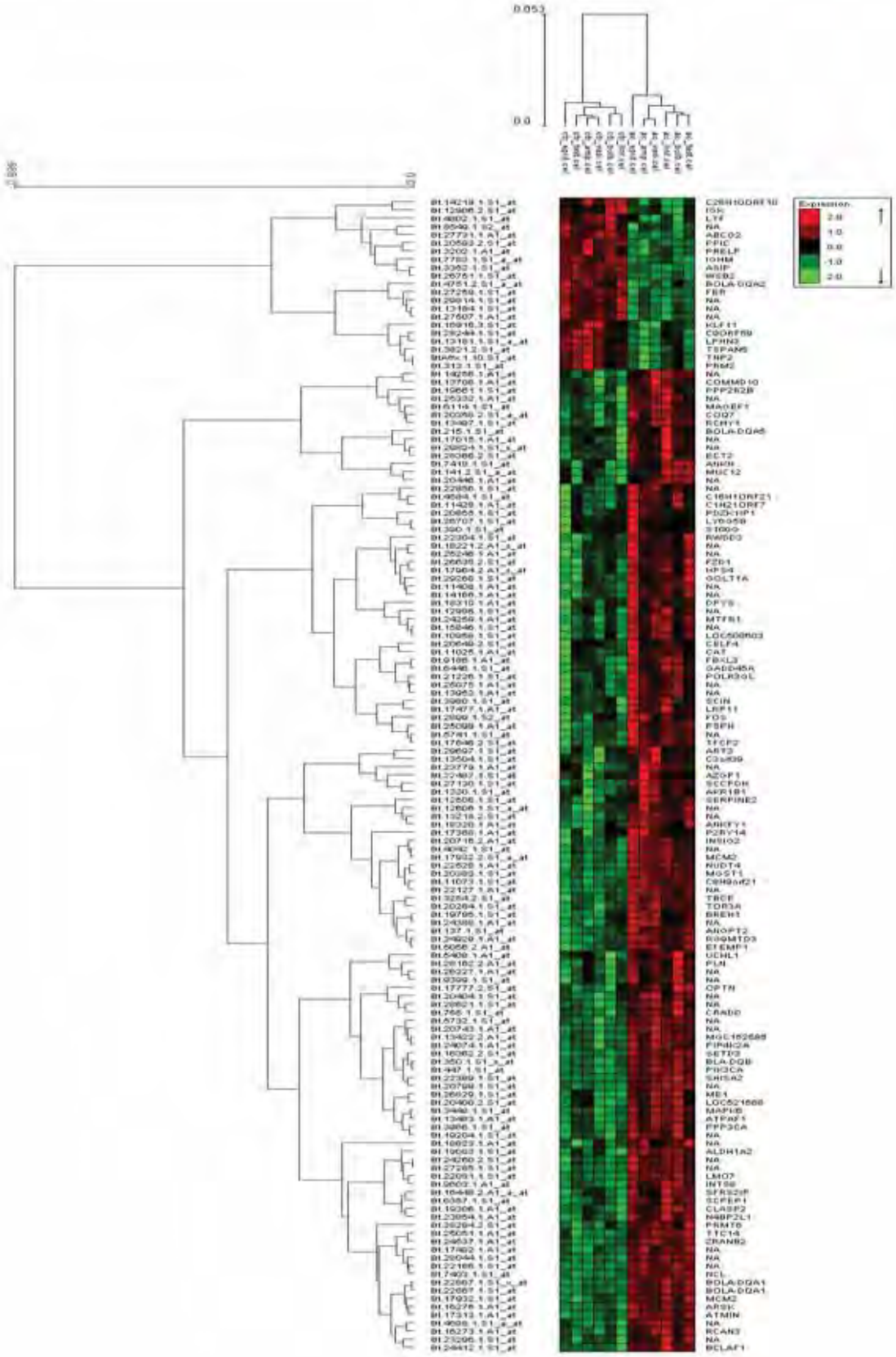
0.053
0.0
Expression
2.0
1.0
0.0
-1.0
-2.0

Fig.2. Condition trees of clustering analysis. The Hierarchical Clustering analysis (HCL method) was performed using Expander (Shamir et al., 2005), and 139 genes that differed significantly. Each colored bar represents a gene. The color represents the level of expression (upregulated in red, and downregulated in green), and the samples (control and infected) information is listed across the top. The gene names are indicated. Note the distinct patterns of altered gene expression between the positive and control animals.

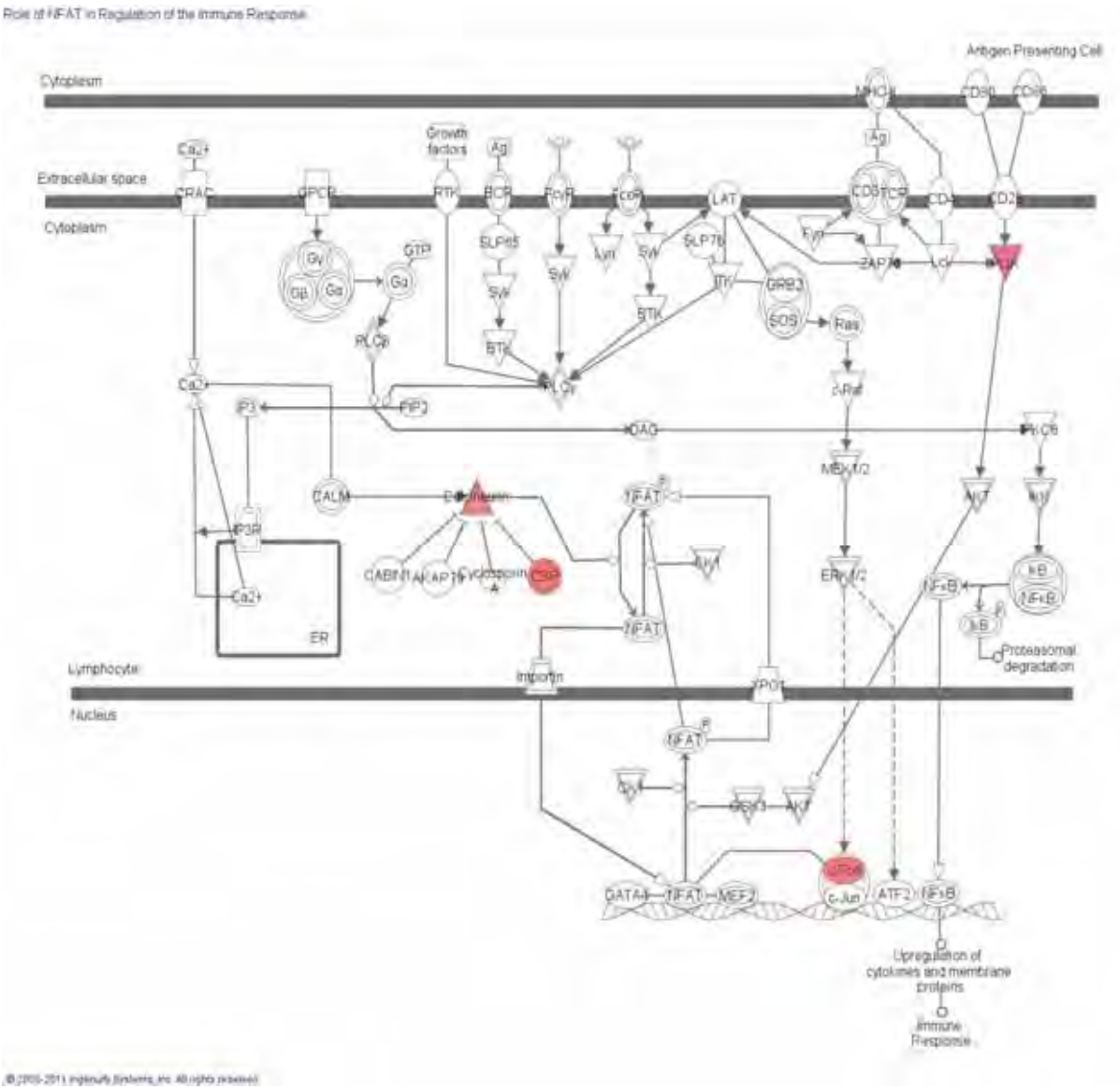

Figure 3. The upregulated genes in infected rams are indicated in red. The blank symbols pertain to genes that were either not present in our array or not differentially expressed. Solid lines indicate a direct linkage among two genes. Lines with arrows indicate that one gene acts on the other, and lines without arrows indicate that the corresponding proteins interact with each other.

Table 3. Canonical pathways associated with the DEGs from 120 dpi.

Ingenuity Canonical Pathways[a]	**p-value**[b]
Inhibition of Angiogenesis by TSP1 (tumor suppressor region 1)	1.73E00
Mechanisms of Viral Exit from Host Cells	1.25E00
PKCθ (protein kinase C theta) Signaling in T Lymphocytes	8.05E-01
Caveolar-mediated Endocytosis Signaling	6.7E-01
FcγRIIB (Fc receptor or CD32) Signaling in B Lymphocytes	6.03E-01
Phospholipase C Signaling	4.97E-01
RhoA (RhoA kinase) Signaling	4.79E-01
Complement System	4.79E-01
NRF2(nuclear factor erythroid 2) mediated Oxidative Stress Response	4.36E-01
Lipid Antigen Presentation by CD1	4.28E-01
Role of JAK2 (Janus Kinase 2) in Hormone-like Cytokine Signaling	4.18E-01
Coagulation System	3.63E-01
Crosstalk between Dendritic Cells and Natural Killer Cells	3.55E-01
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	3.34E-01
Retinoic acid Mediated Apoptosis Signaling	3.25E-01
Endoplasmic Reticulum Stress Pathway	2.99E-01
Role of JAK1 (Janus Kinase 1), JAK2 and TYK2 (tyrosine kinase 2) in Interferon Signaling	2.81E-01

[a] Functional annotation analysis of the differentially expressed genes (DEGs) with the IPA (Ingenuity Pathway Analysis, http://www.ingenuity.com) tools.

[b] P value determined from the average log2 ratio using Bioconductor (http://www.bioconductor.org).

2.4 Comparison of DEGs among collection times (acute, chronic I and chronic II) in R-*B. ovis* infected rams

The Venn diagram (Figure 4) illustrates the number of DEGs obtained in each of the four comparisons, as well as the number of DEGs shared between the comparisons. From the 44 common DEGs (31 known genes) obtained in the comparison performed among the three groups (comparison 4), only 8 had significant biological functions (Table 1). The IPA tool allowed for comparison of the results of different tests. This feature enabled the identification of biological functions and/or enriched canonical pathways common to different analysis. When the results of the three IPA analysis were compared, the biological functions of immune cell trafficking, immunological disease, infectious disease, inflammatory disease, inflammatory response and cellular movement were found as significant for the three test conditions (acute, chronic I and II) and a greater significance was observed for phase chronic II (Figure 5).

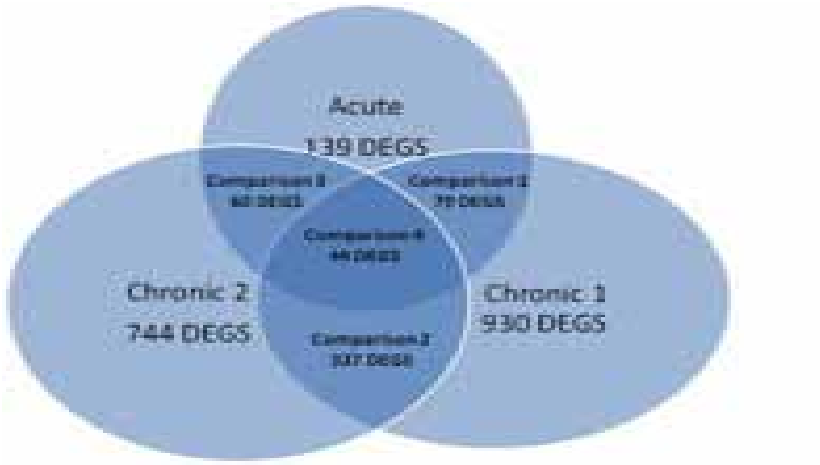

Fig.4. Venn diagram showing the DEGs obtained in each of the four comparisons, and the number of DEGs shared between the comparisons.

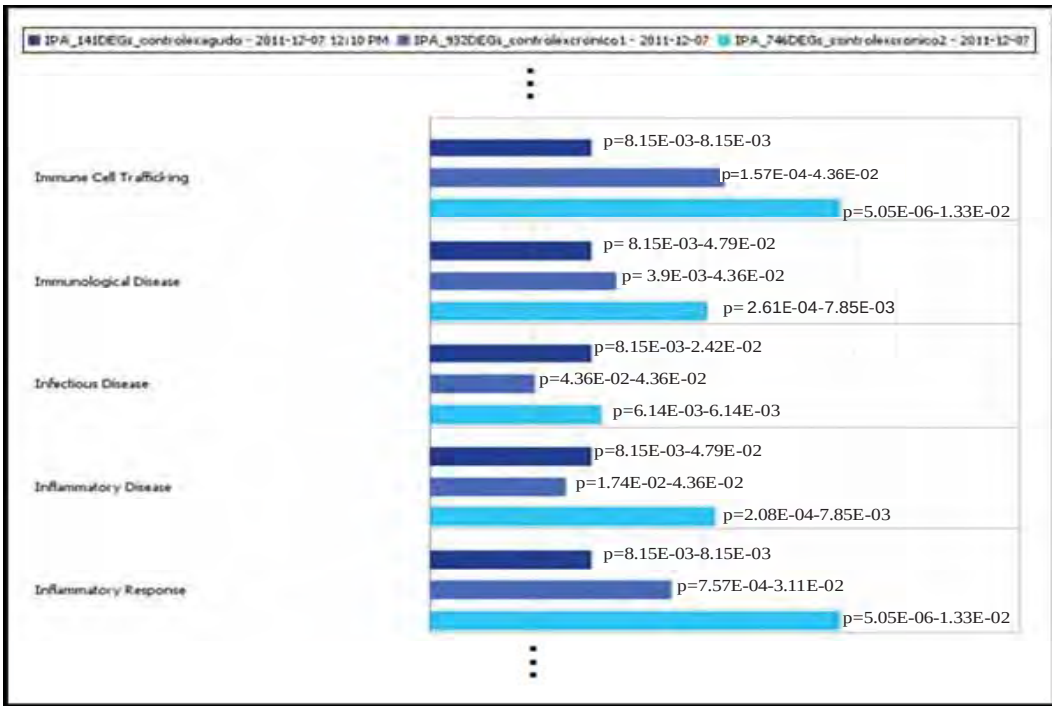

Fig.5. Functional analysis of the phases of infection using IPA (Ingenuity Pathway Analysis - http://www.ingenuity.com/). As shown in the legend, the analysis control X acute is represented in dark blue, control X chronic 1 is visualized with medium blue, and with light blue the control X chronic 2 is demonstrated. The biological functions are significant ($p<0.005$) for three test conditions: acute, chronic 1 and 2.

3. Discussion

3.1 Acute phase of infection (60 dpc)

An association between significant DEGs and the canonical pathways at 60 dpi was demonstrated using the IPA tool, which was indicative of an innate immune response (Table 2). The clinical presentation of ovine brucellosis, characterized by epididymitis and orchitis (Megid et al., 2010), was highlighted by general upregulation of genes in reproductive organs and lymph nodes (Figure 2). Previous studies demonstrated the upregulation of pro-inflammatory genes as IL-1, IL-2 in infected rams (Galindo et al., 2009). The upregulation of DEGs involved with innate immune response in

reproductive tissues and lymph nodes may have been directed toward elimination of the bacteria. The upregulation of genes during the acute phase of infection was suggestive of a general antibacterial host mechanism in response to the intracellular pathogen (Galindo et al., 2009). The presence of genes associated with the IL-6 pathway provided evidence of macrophage activation as described by Salas-Téllez et al. (2005). Activation of IL-12 pathway suggests a response to bacterial infection and enhanced cell-mediated cytotoxicity (Rose et al., 2000). As reported by Galindo et al. (2009), at 60 dpi, R-*B. ovis* infection in rams induces an activation of inflammatory and innate immune response pathways which contribute to development of a chronic R-*B. ovis* infection. By use of the IPA tool, evidence of an anti-inflammatory response against R-*B. ovis* was provided by demonstration of the pathways of macrophage migration inhibition factor (MIF) and IL-10. As reported by Golding et al. (2001), the presence of IL-10 contributes to the control of the intracellular growth of *Brucella* spp.

In this study expression of genes upregulated (BOLA genes: BOLA-DQA1 and BOLA-DQB), during acute infection were associated with the canonical pathways (Tabele 2). Previous studies have reported that expression of BOLA-DQA and BOLA-DQB genes may contribute to the progression of mastitis (Takeshima et al., 2008). BOLA genes, especially the major histocompatibility complex (MHC), genes have been associated with genetic control of immune responses, during which they promote resistance or susceptibility to clinical mastitis (Takeshima et al., 2008). In this study, the major histocompatibility complex class IIDQ alpha 2 (BOLA-DQA2) from the group of Bovine Leukocyte Antigen (BOLA) class II genes which were in the group of downregulated DEGs, are involved in the immune response and were reported previously to be associated to mastitis resistance in dairy cattle (Hou et al., 2011). Downregulation of BOLA-DQA2 during the acute phase infection may represent a

mechanism that R-*B. ovis* for evasion of the immune response in infected rams that was not reported previously. An understanding of the role of BOLA genes in ovine brucellosis may contribute to our understanding of different phases, including susceptibility, chronicity, and resistance disease. During the acute phase of infection, the pathogenicity of *B. ovis* activates the immune response with participation of gene expression, pathways and networks directed primarily toward elimination of the bacteria.

3.2 Chronic phase of infection I (120 dpc)

A great number of DEGs were observed at the chronic phase I, with an increase in downregulated genes. These results are in accordance with Galindo et al. (2009), who reported that *B. ovis* induces downregulation of gene expression in buffy coat cells of infected rams in order to suppress the inflammatory host defenses. In Table 3, the IPA tools through the canonical pathways emphasizes an immune response associated with chronicity of R-*B. ovis* infection. This intracellular bacteria stimulates a cellular response (Vemulapalli et al., 2000), however, an effective response to *Brucella* spp. requires both humoral and cellular immune responses (Ficht & Adams, 2009). The canonical pathway NFAT remained significant until 120 dpi, and this family of transcription factors plays an important role in the immune response (Crabtree and Olson 2002). At 120 dpi the JAK1 pathway was significantly altered, this pathway is an essential signaling protein used by cytokines type I and type II in response to tumors (Jatiani et al., 2010). TYK2 was the first member of the JAK family described, and has been implicated in IFN-α, IL-6, IL-10 and IL-12 signaling (Stark et al., 1998). The genes found in association with the canonical pathway of FcγRIIB receptor provided evidence of protective immune functions. According to Anderson (2003) the Fc

receptor is a protein found on the surface of natural killer cells, macrophages, neutrophils, and mast cells that contributes to the activity of cytotoxic cells trough the antibody-dependent cell-mediated cytotoxicity. IGF-1 canonical pathway was significant in this study, upregulation of IGF1 gene has been related to macrophages activation, thus contributing to pathogenesis of R-*B. ovis* in rams (Mora et al., 2006; Galindo et al., 2009). Upregulation of genes associated with the CTLA4 canonical pathway suggested a tentative response to suppress the immune response to R- *B. ovis* because this signaling is transmitted as an inhibitory signal to T cells (Fernández-Mestre et al., 2009). The upregulated genes associated to SAPK/JNK canonical pathway (significant at both 60 and 120 dpi) demonstrated a cellular commitment to eliminate R-*B. ovis.* This signaling is responsible for the nuclear production of NF-kappaB, TNF-α and IL-2 during T lymphocyte activation, which, in turn, regulates cell survival, apoptosis, and proliferation (Nishina et al., 2004). The canonical pathways VEGF remained activated at chronic phase I of infection. VEGF genes are associated with inflammatory reaction induced by intracellular bacterial infection (Sato et al., 2007). The JAK-STAT canonical pathway seems to be relevant during acute phase of infection and chronic phase I. This pathway is a major signaling alternative to the second messenger system that is activated by a signal from interferon, interleukin, growth factors, or other chemical messengers that act in basic cell functions, such as cell growth, differentiation and death. Failure of JAK-STAT pathway can result in immune deficiency syndromes and cancer (Aaronson and Horvath, 2002). At the chronic phase I of infection upregulation of BOLA genes (BOLA-DQA1 and BOLA-DQB) was observed in infected tissues, and suggested a role of these genes during ovine brucellosis. The BOLA-DQA2 gene (MHC class I heavy chain) remained downregulated after the acute phase of infection. The upregulation of genes linked in

canonical pathways associated with immune responses may represent an antibacterial mechanism of the host for elimination of R-*B. ovis* infection.

3.3 Chronic phase of infection II (240 dpc)

At this stage of infection a strong cellular commitment was noticed, demonstrating the chronicity of ovine brucellosis. The chronicity of B. *ovis* infection is mainly evidenced by the biological functions inflammation and coagulation. According to Bergmann and Hammershmidt (2007), the interplay between blood coagulation and inflammation is essential to the host defense against intracellular infectious agents, however, this interpolation favors dissemination of bacteria within the host and contribute to avoid the immune response. At 240 dpi activation of inflammatory and LPS/IL-1 inhibition pathways were observed. Changes of gene expression during *B. ovis* late infection may be transient in order to extend the intracellular life of the pathogen, and may occur by upregulation of genes involved in bacterial phagocytosis and downregulating of genes resulting from a protective host defense mechanism (Galindo et al., 2009). Of the 86 downregulated genes observed, the analysis emphasizes downregulation of cytokine IL-2, BOLA and aquaporin. Galindo et al. (2009) reported a Th1 profile and a suppression of Th2-type response at 60 dpi. During chronic infection II a remarkable upregulation of pro-inflammatory and phagocytosis genes as BOLA-DQA1, BOLA-DQB and annexin was occurred, demonstrating a polarization of Th1 response. A Th2 response was also observed in the DEGs studied. The aquaporin genes are involved in the transport of small solutes and water channels, and alteration of these genes has been associated to several human diseases (Agre and Kozono, 2003). Dowregulation of the aquaporin genes may be a finding not reported previously of the mechanisms of *B. ovis* during the onset of the disease. The results from the gene profile results in the late phase of

infection suggested that *B. ovis* employs mechanisms that facilitate the persistence on the host.

4. Validation of the microarray analysis

Of the 8 genes that were chosen from the microarray experiment, five were correlated with the data generated from qRT-PCR (Table 4). Differences in the infected/uninfected ratio obtained by both methods may have resulted from variations in the sensitivity of the methods and from animal-to-animal variations, which were more evident in the qRT-PCR analysis with a larger number of animals (Galindo et al., 2009). The analysis during the acute phase of infection on the microarray study suggested the relevance of the ataxia telangiectasia-mutated ATMIN (ATM interactor) gene. The ATM gene acts as a tumor suppressor, activated by DNA damage and interacts with a broad network of proteins as the tumor suppressor (p53), DNA repair factors (RAD50, RAD51, GADD45) and other signaling molecules (NF-kB). In addition to regulating DNA repair and the cell cycle, ATM can also trigger apoptosis in cells exposed to radiation (Mc Kinnon, 2004). ATM gene may be activated to cause apoptosis and release the bacteria from infected cells, thus contributing to immune response against R-*B. ovis*. The genes. RCHY1 (ring finger and CHY zinc finger domain containing 1), ANKFY1 (ankyrin repeat and FYVE domain containing 1) and EFEMP1 (EGF-containing fibulin-like extracellular matrix protein 1) were also highlighted by the analysis during chronic phase I of infection. The RCHY1 gene is expressed at higher levels in liver, testis and heart promoting p53 degradation. Higher levels of expression of RCHY1 have been associated to lung tumorigenesis (Beitel et al., 2002). Ankyrin proteins have been associated with a number of human diseases, including the cell cycle inhibitor p16, which is associated with cancer (Mosavi et al., 2004). EFEMP1 gene has been shown to

promote tumor growth in human adenocarcinoma (Camaj et al., 2009). The genes RCHY1, ANKFY1, and EFEMP1 were identified as genes involved in cancer, which could be relevant in ovine brucellosis because of the role they play in the initiation of apoptosis, inhibition of cell cycle and progression of infection. ZRAB2 was validated at chronic phase II and this gene was implicated in regulation of mRNA processing (Nguyen et al., 2011). When the results of the three IPA analysis were compared (Figure 5), the experimental infection demonstred a natural evolution of the ovine brucellosis, where the biological functions: immune cell trafficking, immunological disease, infectious disease, inflammatory disease, inflammatory response and cellular movement were phase to phase increasing their significance during the infection; this analysis proves the persistence and chronicity of *B. ovis*.

This study represents the first analysis of microarray gene expression in different tissues of rams infected with of R-*B. ovis* at various times during infection (60 dpi (acute phase), 120 dpi (chronic phase 1), and 240 dpi (chronic phase 2), and many of the genes identified require futher study. Future studies should be focused on the 31 common genes altered at the three times of infection using the PCR array tool. The microarray used for this study was designed for gene expression in cattle, and validation of these common DEGs will be necessary to avoid the underestimation of gene expression involved in the pathogenesis of R-*B. ovis* in rams.. These results expand the pathogenesis knowledge of *B. ovis* R strain infection in rams and suggest new genes and pathways to be investigated in the future.

Table 4. Genes validated through qRT-PCR

Genes	Relative fold-change of genes analysed by qRT-PCR.	Relative fold-change of genes analysed by microarray	Spearman's correlation test (r-value)
ZRANB2 (C2)	5.880 (±0.07; p=0.03)	6.33(±0.29)	r=0.77
RCHY1 (C1)	1.38 (±1.00;p=0.04)	1.32 (±0.11)	r=0.85
ANKFY (C1)	1.46(±0.54;p=0.03);	1.35(±0.11)	r=0.9
ATMIN (A)	6.35(±0.04;p=0.01)	6.25 (±0.51)	r=0.8
EFEMP1 (C1)	1.76(±0.04;p=0.02)	1.88(±0.17)	r=0.8

A: acute phase; C1: chronic phase 1; C2: chronic phase II

5. Experimental Procedures

5.1 Experimental infection

Twelve, 1 year-old Santa Inês rams, confirmed to be negative for brucellosis, Visna-Maedi, toxoplasmosis and neosporosis, were selected for the study. The rams were kept in an isolated pen and experimentally-infected with the R-*B. ovis* PA strain (ATCC25840) (Blasco et al., 1990), provided by Dr. Renato L. Santos (Universidade Federal de Minas Gerais, UFMG, Belo Horizonte, Minas Gerais, Brazil). Experiments were conducted with the approval of Ethical Committee of UNESP (Ethics committee protocol nr.: 69/2008). The R-*B. bovis* strain was cultured on blood agar base plates, and incubated for 48h at 37^{o}C and 10% CO_2. Bacteria were then washed with 10mM phosphate buffered saline (PBS; pH 6.8), and the concentration was adjusted using a spectrophotometer (550nm). The rams were then infected with a total of 2.2 x 10^9 *B. ovis* CFU in 60 µl administered in two doses (30 µl administered conjunctivally and 30 µl intrapreputially). Uninfected (control) rams were inoculated in the same manner with sterile distilled water. Rams were then assigned four to groups of 3 rams each: (1) control (0 dpi), (2) acute phase (60 dpc) (3) chronic phase I (120 dpc) and (4) chronic

phase II (240 dpc). From each group, samples of epididymus, testicles, ampolae, vesicular glands, bulbourethral glands, and a pool of lymph nodes (inguinal and scrotal) were collected from each ram (Fig. 1). Gene expression changes associated to *B. ovis* infection were determined using using microarray analysis and compared between the infected control groups. All tissues were tested by PCR for *B. ovis* infection (Manterolla et al., 2003). After 15 dpi, infection in rams was also confirmed by serology using the Agar Gel Immunodiffusion (AGID; Instituto de Pesquisas Veterinárias Desidério Finamor – IPVDF, Eldorado do Sul/RS, Brazil).

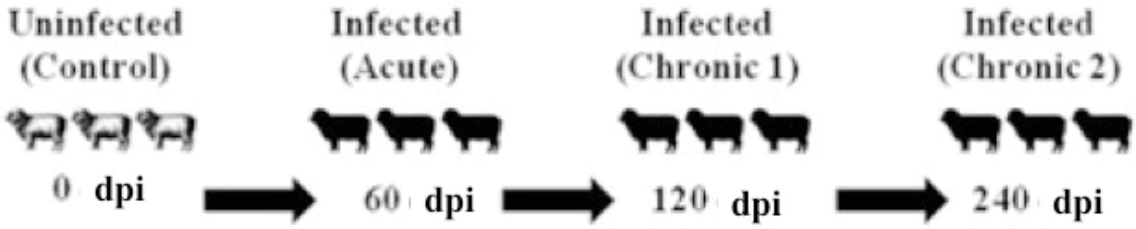

Fig.1. Experimental design. Differential expression of genes from infected rams was compared with control (0 dpc) by microarray analysis during the course of infection.

5.2 Microarray hybridization and analysis

Total RNA was isolated using Invisorb® Spin Tissue RNA Mini Kit (Invitek Gmbh, Germany) according to manufacturer's instructions from tissue samples collected at 0, 60, 120, 240 dpi, and purified with the RNeasy Mini-kit (Qiagen, Mississauga, ON, Canada). Total RNA was quantified by absorbance at 260 nm. The quality of the RNA preparations was confirmed by electrophoresis and the Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Only samples with a preserved rRNA ratio (28S/18S), no evidence of RNA degradation and values ≥ 8 at the Bioanalyzer were used in the microarray hybridization and qPCR studies. Expression measurements of

23,000 genes were performed with the Affymetrix GeneChip® Bovine Genome Array (Affymetrix, USA) and the RNA labeling and hybridization protocols were carried out according to manufacturer's instructions. After array scanning, quality control was performed using the GCOS software (Affymetrix, USA) according to the manufacturer's recommendations. The quality control also was verified using Expression Console (http://www.affymetrix.com/browse/level_seven_software_products_only.jsp?productId=131414&categoryId=35623#1_1). The unwanted variables (batch effects) were removed using ComBat methodology (http://jlab.byu.edu/ComBat/Abstract.html) (Johnson et al., 2007). The IQR (Inter-Quartile-Range) filter available at R/Bioconductor (Irizarry et al., 2003) was then applied. Gene expression values were obtained using the three-step Robust Multi-array Average (RMA) pre-processing method, implemented in the Affy package from R/Bioconductor (Irizarry et al., 2003). In order to select DEG's the non-parametric RankProd method was used with a p-value equal or greater to 0.05 and adjusted for FDR (False Discovery Rate) (Benjamini & Hochberg, 1995; Hong et al., 2006), a method which uses hierarchical clustering (HCL)to group data with the Pearson correlation coefficient to calculate the distance and linkage between groups (available at http://acgt.cs.tau.ac.il/expander/index.html) (Shamir et al., 2005). In order to perform a functional annotation analysis of DEG's, IPA (Ingenuity Pathway Analysis, http://www.ingenuity.com) tools were used, considering the default parameters: molecules per network=35; networks per analysis=25; and direct relationships between genes and the "Ingenuity Expert Information" Data Source, including the new option of "Ingenuity Expert Assist Findings" in which the information was manually reviewed and curated from full-text

scientific publications. The microarray gene sequences were deposited in the NCBI Gene Expression Omnibus (GEO) under the platform accession number GSE35615.

5.3 Validation of microarray expression using real-time qRT-PCR

The total RNA extracted from experimentally-infected ram tissues collected at 0, 60, 120 and 240 dpi was analyzed by qRT-PCR using gene-specific primers and the iScript One-Step RT-PCR Kit with SYBR Green and an iQ5 thermal cycler (Bio-Rad, Hercules, CA, USA) following manufacturer's recommendations. The mRNA levels were normalized against sheep β-actin and GAPHD and the ratio between infected (60, 120 and 240 dpi) and uninfected (0 dpi) values calculated. Data were normalized to a calibrator sample using the ΔΔCt method with correction for amplification efficiency (Pfaffl, 2001). The validation of microarrays data was done by comparisons between DEGs. Comparison 1: acute vs chronic I, comparison 2: chronic I vs chronic II, comparison 3: acute vs chronic II and comparison 4: comparison 1 vs comparison 3. To validate the microarray data, 8 common DEGs between the three stages of infection (comparison 4) and with the most significant biological functions (IPA tools) were chosen for analysis by qRT-PCR (Table 1). The correlation between microarray and qRT-PCR assays for the infected and control rams was calculated using Spearman's correlation test.

Table 1. Primer sets and qPCR conditions used for validation of microarray experiment.

Gene description	Genbank accession number	qPCR annealing conditions
Arylsulfatase family, member K (ARSK)	NM_001035400	55^0C, 30s
Ring finger (RCHY1)	NM_001083754	55^0C, 30s
Ankyrin (ANKFY1)	XM_606825	55^0C, 30s
EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1)	NM_001081717	55^0C, 30s
ATM interactor (ATMIN)	XM_001249565	55^0C, 30s
Zinc finger, RAN-binding domain containing 2 (ZRANB2)	NM_001105346	55^0C, 30s
Histocompatibility complex, class II, DQ alpha, type 1(BOLA-DQA1)	NM_001013601	55^0C, 30s
MHC class II antigen (BLA-DQB)	NM_001034668	55^0C, 30s
Ovis aries glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_001190390	56^0C, 30s

Conflict of interest statement

All authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence their work.

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V.DISCUSSÃO GERAL

Transcrevendo PAPPAS (2010), “entender a patogênese das *Brucella* spp. é semelhante a olhar pelo buraco da fechadura por um longo período e posteriormente, quando a porta se abre lentamente, o panorama de uma sala escura emerge, mas ainda precisa de muita luz”.

Sob condições experimentais de infecção, na brucelose ovina o agente permanece confinado próximo aos locais de entrada, por duas ou três semanas, em órgãos genitais e glândulas acessórias do aparelho reprodutor, para depois ocorrer a bacteremia, seguida de infecção do sistema mononuclear fagocitário (SFM) e linfonodos. Em animais experimentalmente infectados, a bactéria foi isolada do fígado, dos rins, do baço, dos testículo, do epidídimos, da vesícula seminal, das glândulas bulbouretrais, das ampolas e dos linfonodos regionais (SANTOS et al., 2005a).

Recentes avanços demonstram que *Brucella* spp. geram somente uma resposta inflamatória moderada provavelmente como resultado de tentativa de evasão e supressão da RI hospedeira (XAVIER et al., 2010). A natureza intracelular do agente estimula uma RIC (VEMULAPALLI et al., 2000) tipo Th1 e Th2; secreção de IL-2, INF-γ, TNF-α, principalmente, é observada na resposta Th1 envolvidas na RIC e IL-4, IL-5, IL-6, IL-10 por Th2 promovendo a RIH (SALAS-TÉLLEZ et al., 2005). Estudos até hoje provaram que tanto a RIC como a RIH participam na proteção contra a brucelose, pela combinação de anticorpos (Ac) e RIC (ARAYA et al., 1989; FICHT; ADAMS, 2009).

Segundo Tizard (2009), quando as células de defesa (macrófagos, células dendríticas, mastócitos) são expostas aos PAMPS das bactérias através dos receptores PRRs tipo TLRs, elas secretam diversas citocinas, principalmente IL-1α, TNF-α, assim como IL-6, IL-12 e IL-18, desencadeando uma inflamação e uma resposta de fase aguda.

GALINDO et al. (2009), utilizaram a mesma cepa de *B. ovis* PA que a utilizada neste estudo, e demonstraram pela tecnologia de microarranjo que até 60 dpi uma maior expressão de genes envolvidos com a ativação da resposta inflamatória e fagocitose e menor expressão dos genes envolvidos com os mecanismos de proteção pode ser observada; estes aspectos favorecem a cronicidade, mantendo predominantemente um perfil de RIC com LTh1.

Apesar de outras pesquisas anteriores terem analisado o genoma de *B. ovis* (TSOLIS et al., 2009), bem como a expressão gênica em camada de células brancas de carneiros infectados com *B. ovis* (Galindo et al., 2009), os trabalhos aqui apresentados constituem os primeiros a avaliar a expressão gênica de citocinas por RT-qPCR e por microarranjos em tecidos de carneiros infectados experimentalmente até 240 dpi.

Tanto no estudo de citocinas (Trabalho cientifíco 1) como na pesquisa com a tecnologia de microarranjos (Trabalho científico 2) podemos comprovar a cronicidade da *B. ovis,* caracterizada por sua detecção por PCR por até 240 dpi nos tecidos avaliados. A RI foi evidenciada com aumento de expressão de citocinas e vias relacionadas com a RI, demonstrado que a *B. ovis* estimula uma RI do hospedeiro, mas não o suficientemente eficaz para eliminá-la. A RI prolongada e o escape da RI do hospedeiro por parte da *B. ovis* pode ser sugerida pelas diferenças no LPS da *B. ovis,* que podem afetar a interação com os TLRs da RI inata (TSOLIS et al., 2009). *Brucella* spp*.* induzem uma fraca resposta inflamatória retardando uma RI efetiva através de um LPS pouco imunogênico que escapa de detecção dos TLRs da RI inata (LAPAQUE et al., 2006), o que favorece sua cronicidade nos animais infectados.

O aumento das citocinas aos 30 dpi em epidídimos (IL-6, IL-1β, IL-1α), testículos (INF-γ, IL-12), glândulas bulbouretrais (IL-6, TNF-α) e ampolas (IL-10, IL-1β, IL-1α, INF-γ) foi estatisticamente significativo, sugerindo com a PCR/sorologia positiva dos tecidos a confirmação do desenvolvimento da enfermidade. De acordo com Blasco et al. (1987), a brucelose ovina é clinicamente caracterizada por epididimite, orquite e infertilidade nos machos. A colonização de testículos e epidídimos é esperada, uma vez que *B. ovis,* após sua penetração, coloniza linfonodos regionais, produz bacteremia com infecção do sistema reticuloendotelial (SER) e finalmente coloniza o trato genital e glândulas acessórias por volta dos trinta dias (BIBERSTEIN et al., 1963; RIDLER; WEST, 2011). O aumento da expressão de IL-1α, IL-1β, IL-6, IL-10, IL-12, INF-γ e TNF-α nos tecidos reprodutivos (Figura 2, trabalho científico 1) caracteriza uma tentativa de eliminação da *B. ovis,* já que uma RI efetiva contra a brucelose envolve tanto RIH (Th2) quanto RIC (Th1) (REFIK et al., 2004).

A detecção de IL-6 (epidídimos, glândulas bulbouretrais) e IL-12 (testículos) está diretamente ligada a ativação e tentativa de eliminação da *B.*

ovis, já que é relatado que a administração de IL-12 ativa a produção INF-γ por células Th1 e aumenta a imunidade protetora (ROSE et al., 2000).

O fato de não ter sido observado aumento de expressão de citocinas tanto por tecidos não reprodutivos como em glândulas vesiculares pode ser explicado por ausência de produção de TNFα por células infectadas por *Brucella* spp., o que, como consequência de seu mecanismo em cascata, não induz outras citocinas (CARON et al., 1994a, b).

A baixa expressão ou a não significância estatística das citocinas em baço, fígado, rins e glândulas vesiculares pode ser justificada por uma possível teoria discutida por Dornand et al. (2002), que propõem uma hipotética via da RI contra brucelose em humanos, na qual a expressão de Omp25 na superfície da bactéria levaria à não produção de TNF-α e como consequência diminuiria a ativação dos macrófagos e NK, e inibiria a produção de INF-γ e citotoxidade mediada pela NK. Devido à ausência de INF-γ/ TNF-α, o bloqueio da ativação de macrófagos resulta na inibição de produção de IL-12 e na inibição do desenvolvimento de uma resposta Th1 (LT $CD4^+$ e LT $CD8^+$ citotóxico) necessária para total eliminação da bactéria.

Por qPCR aos 60 dpi visualizou-se uma diminuição de expressão das citocinas estatisticamente significativa em todos os órgãos avaliados com exceção das glândulas bulbouretrais (Figura 2, trabalho científico 1). O aumento de expressão de TNF-α e IL-6 nas glândulas bulbouretrais (Figura 2, trabalho científico 1) pode ser confirmado em estudos em camundongos e em macrófagos, nos quais as citocinas produzidas durante a infecção contribuíram para o controle do crescimento intracelular de *B. abortus* (ZHAN et al., 1993; DORNAND et al., 2002). TNF-α é a primeira citocina a ser produzida em camundongos e desencadeia a imunidade específica contra patógenos intracelulares e age na indução precoce de IL-12 e INF-γ (ZHAN et al., 1996). Neste experimento, por qPCR nos órgãos reprodutivos e acessórios, visualizou-se expressão aumentada de TNFα, IL-6 e IL-12, sugerindo ativação de macrófagos na tentativa de eliminação do microrganismo.

Pela tecnologia de microarranjo, aos 60 dpi, utilizando a ferramenta IPA tool (*Ingenuity Pathway Analysis*) as funções biológicas e as vias canônicas relacionadas aos genes diferencialmente expressos (*Differentially Expressed*

Genes) (DEGs) indicaram uma RI inata contra a infecção por *B. ovis*. Esta RI é comprovada pelas funções principais (*top functions)* e vias (*pathways)* (Tabela 2, trabalho científico 2). A ação antiinflamatória de *B. ovis* também é visualizada nos tecidos reprodutivos e *pool* de linfonodos através das *vias* da IL-10 e fator de inibição de migração de macrófagos (*macrophage migration inhibition factor)* (MIF).

Apesar da diminuição na expressão das citocinas por qPCR aos 60 dpi (Figura 2, trabalho científico 1), as vias canônicas e funções biológicas das citocinas foram destacadas pela análise funcional por meio do programa IPA. Na análise funcional dos microarranjos, os genes (DEGs) com expressão aumentada nos tecidos reprodutivos e *pool* de linfonodos estão relacionados com as funções principais associadas a doença infecciosa, doença imunológica e doença inflamatória principalmente, indicando uma tentativa do hospedeiro de eliminar a bactéria, o que é reforçado por Galindo et al. (2009), que declaram que o aumento da expressão de genes da RI sugere um mecanismo antibactericida do carneiro em resposta ao patógeno intracelular.

A produção de TNF-α aos 120 dpi (Figura 2, trabalho científico 1) e as sinalizações por receptores de TNF-α, de citocinas e de células de defesa, foram significantes através da ferramenta IPA *tool* até 120 dpi; sabe-se que o TNF-α é a primeira citocina a ser produzida em camundongos infectados com *B. abortus,* desencadeia a imunidade específica contra patógenos intracelulares e age na indução precoce de IL-12 e INF-γ (ZHAN et al., 1996).

O papel importante da queda na expressão de IL-12 e da IL-6 a partir dos 30 dpi (Figura 2, trabalho científico 1) pode ser explicado através da molécula MyD88 que é um adaptador geral para TLRs e de algumas interleucinas que é requerida para o controle da replicação de *Brucella spp*. O MyD88 é responsável pela maturação de DC e produção de IL-12 e TNF-α por macrófagos. A falta da IL-12 é crítica no que se refere à susceptibilidade na infecção por *Brucella* spp. MyD88 é também usada por outras vias de sinalização como a da IL-1 e IL-18 (WEISS et al., 2005; COPIN et al., 2007; MACEDO et al., 2008).

Aparentemente o aumento de expressão da IL-12 em testículos aos 30 dpi poderia ser justificada como uma tentativa de evitar a multiplicação da *B.*

ovis. A IL-12 é uma citocina heterodímera produzida principalmente por fagócitos e células apresentadoras de antígenos (APC) que atuam na RI inata e adaptativa em resposta a infecção bacteriana. Células NK se proliferam em resposta a IL-12 e também melhoram a citotoxidade mediada por células induzindo uma maior produção de INF-γ. Nos tecidos reprodutivos e nas glândulas acessórias dos animais estudados observou-se produção de IL-12 e de INFγ (Figura 2, trabalho científico 1) demonstrando ativação da resposta imune. A produção de INF-γ por células NK e T promove a produção de IL-12 por macrófagos e expansão de células Th1 $CD4^+$, e este *feedback* pode ser antagonizado pela IL-10 que é produto de células Th2 (ROSE et al., 2000), o que pode ser demonstrado em ampolas (Figura 2, trabalho científico 1).

O pico de IL-6 em glândulas bulbouretrais aos 120 dpi (Figura 2, trabalho científico 1) também é reforçado por sua própria via (Tabela 2, trabalho científico 2) no estudo de microarranjos, que sugere a ativação de genes de citocinas pró-inflamatórias e resposta imune adquirida (SALAS-TÉLLEZ et al., 2005). As vias canônicas e as redes biológicas aos 120 dpi sugeridas no trabalho científico 2 (Tabela 3) sugerem ativação dos dois braços da RI: a RIC e RIH. A ativação da RI pode ser explicada pela cronicidade da infecção por *B. ovis* e demonstrada pelo aumento da expressão de IL-6 em epidídimos (Figura 2, Trabalho científico 1), pelo pico de IL-6 em glândulas bulbouretrais (Figura 2, Trabalho científico 1) e pelas vias canônicas mais específicas em relação a RIC (Tabela 3, trabalho científico 2). *B. ovis,* devido a sua vida intracelular, estimula RIC (VEMULAPALLI et al., 2000), entretanto, para que uma RI seja eficaz contra *Brucella* spp., são necessários os dois tipos de RI (FICHT; ADAMS, 2009), o que pode ser confirmado pela ativação da via canônica do fator de transcrição *Janus Kinase* (JAK) (Tabela 3, trabalho científico 2), importante na sinalização de citocinas Th1 e Th2 (JATIANI et al., 2010).

A queda de expressão de citocinas (Figura 2, trabalho científico 1), com exceção de TNF-α em glândulas bulbouretrais, pode ser sugerida pela ativação da via canônica CTLA4 (*Cytotoxic T-Lymphocyte Antigen 4*) trabalho científico 2; segundo Fernández-Mestre et al.(2009), a utilização desta via pela *B. ovis* pode suprimir a RI a partir de sinais inibitórios para LT. A ativação de vias canônicas associadas a RI (Trabalho científico 2) pelo programa IPA aos 120

dpi confirma a tentativa de resposta do hospedeiro de eliminar o patógeno intracelular.

Aos 240 dpi existe uma queda generalizada das citocinas nos tecidos que foi estatisticamente significativa, com exceção de IL-6 em epidídimos e de TNF-α em glândulas bulbouretrais (Figura 2, trabalho científico 1). A IL-6 indica ainda uma tentativa de eliminação da bactéria; e em macrófagos em fase tardia de infecção pode estimular a produção de Ac via LB (SALAS-TÉLLEZ et al., 2005). A importância da IL-6, IL-1α, TNF-α e INF-γ nos carneiros do nosso experimento é reforçadas pelo estudo de Cheers (1998), que demonstra a importância do TNF-α no início da infecção ativando macrófagos ou estimulando NK a produzir INF-γ. A menor expressão de citocinas nos outros tecidos pode ser explicada pelo fato de que bactérias do gênero *Brucella* possuem fatores de virulência que evitam a RI visando aumentar sua sobrevivência (SELEEM et al., 2008) e inibir o processo de apresentação de antígenos (BARRIONUEVO et al., 2008), gerando falha em sua eliminação favorecendo a sua disseminação para órgãos do SRE, com linfadenopatia regional (ENRIGHT et al., 1990).

De acordo com Ridler e West (2011), *B. ovis* possui localização restrita aos órgãos reprodutivos/acessórios, e este tropismo poderia ser sugerido pelos resultados do trabalho científico 1, em que a menor expressão dos genes inflamatórios observada nos animais infectados por *B. ovis* poderia explicar sua persistência e cronicidade. Entretanto, parece que a *B. ovis* promove um aumento dos genes proinflamatórios (GALINDO et al., 2009), enquanto outras *Brucella spp.* ocasionam diminuição da expressão de genes proinflamatórios (BARRIONUEVO et al., 2008; MARTIROSYAN et al., 2011).

No trabalho científico 2, demonstrou-se, pela análise funcional, RI persistente causada pela cronicidade de *B. ovis*. Brucelas possuem estratégias de evitar a detecção pelo sistema imune e promover uma infecção prolongada no interior das células modificando a expressão dos genes, incluindo a habilidade de sobreviver em vesículas acidificadas, alterando a apoptose de macrófagos, prevenindo a fusão do fagolisossomo e levando a uma menor ativação dos mecanismos de proteção e da inflamação (RAJASHEKARA et al., 2006). A confirmação de vias de RIC e RIH associadas aos DEGs

correspondente a 240 dpi confirmou a necessidade de ativação de ambas as RI sugerida por Ficht e Adams (2009) no combate a infecção por *Brucella* spp.

No principal estudo de expressão gênica em carneiros infectados com *B. ovis,* Galindo et al. (2009) analisaram, pela primeira vez em leucócitos dos animais infectados, 600 genes de ruminantes (pela a tecnologia de microarranjo no *chip* RIGUA-Ruminant Immuno-inflammatory Gene Universal Array da Universidade de Edinburgh, Escócia, Reino Unido) relacionados com a RI e inflamação em carneiros infectados com *B. ovis* aos 30 e 60 dias pós-infecção (dpi). Os resultados demonstraram que existe ativação da resposta inflamatória e da RI inata, com expressão aumentada dos genes de fagocitose, dos mecanismos de proteção do hospedeiro e expressão diminuída de receptores celulares, o que contribui para a cronicidade da infecção por *B. ovis*.

A utilização de *chips* de hibridização heterólogos para estudo de expressão gênica de uma determinada espécie em outra espécie é frequentemente descrito (GALINDO et al., 2009; PARISET et al., 2009). No presente estudo foi utilizado um *chip* universal para bovinos Affymetrix GeneChip® Bovine Genome Array (Affymetrix, USA), que pesquisa 23.000 genes; os resultados de comparação entre todos os DEGs de todos os momentos sugeriu a ativação das funções biológicas relativas a tráfego de células imunes, doença imunológica, doença infecciosa e movimento celular durante a infecção experimental, demonstrando a cronicidade, o comprometimento celular e a continuidade da tentativa de eliminação da *B. ovis.*

VI.CONCLUSÕES GERAIS

1. Aos 30 dpi houve expressão aumentada das citocinas em epidídimos (IL-6, IL-1β, IL-1 α), testíclos (INF-γ, IL-12), glândulas bulbouretrais (IL-6, TNF-α) e em ampolas (INF-γ, IL-10, IL-1β, IL-1α), comprovando a ativação da resposta imune induzida pela *B. ovis*;
2. Com o decorrer da infecção (60 dpi) houve um decréscimo na expressão de citocinas demonstrando uma evasão da resposta imune, com exceção de glândulas bulbouretrais, o que favoreceu a persistência da *B. ovis*;
3. Durante a fase crônica de infecção (120 e 240 dpi) a *B. ovis* induziu expressão menor das citocinas em epidídimos (IL-1β, IL-1α), testíclos (INF-γ, IL-12) e ampolas (INF-γ, IL-10, IL-1β, IL-1α), com exceção das glândulas bulbouretrais (IL-6 and TNF-α) e epidídimos (IL-6) ainda numa tentativa de eliminação;
4. A avaliação das citocinas sugere uma ativação da RI aos 30 dpi, com um decréscimo da expressão com desenvolvimento da infecção, sugerindo um mecanismo de escape da RI; no final da infecção foi demonstrada uma pequena ativação de citocinas Th1 e Th2, confirmando resposta imune celular e humoral ineficazes para eliminar persistência da *B. ovis*; este perfil de resposta imune é devido à persistência da infecção;
5. O estudo demonstrou a primeira análise do perfil de expressão gênica de citocinas por meio de qRT-PCR em tecidos reprodutivos e não reprodutivos de carneiros experimentalmente infectados com cepa de *B. ovis* PA até 240 dpi;
6. A análise de microarranjos sugere que a *B. ovis* induza a ativação das redes biológicas e vias canônicas relacionadas com: tráfego de células imunes, doença imunológica, doença infecciosa, doença inflamatória, resposta inflamatória e movimento celular, comprovando seu carácter de doença infecciosa crônica;
7. Na fase aguda de infecção (60 dpi) a análise de microarranjos sugere o gene da ataxia telangiectasia-mutated ATMIN (ATM interactor) como possível relevante na apoptose da célula;

8. Na fase crônica 1 (120 dpi) de infecção os genes RCHY1 (ring finger and CHY zinc finger domain containing 1), ANKFY1 (ankyrin repeat and FYVE domain containing 1) e EFEMP1 (EGF-containing fibulin-like extracellular matrix protein 1) foram validados e sugerem participação em vias relacionadas a apoptose, inibição do ciclo celular e persistência da infecção, com possível importância na patogênese da brucelose ovina;
9. O gene ZRAB2 (Zinc finger, RAN-binding domain containing 2) foi relevante na fase crônica 2 (240 dpi) de infecção; entretanto o ZRAB2 é relacionado com aumento do processamento de RNAm, e sua relevância em fases avançadas da brucelose ovina precisa ser mais estudada;
10. O estudo representa a primeira análise geral de expressão gênica por meio de microarranjos em tecidos reprodutivos e pool de linfonodos de carneiros experimentalmente infectados com cepa de *B. ovis* PA até 240 dpi.

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VIII. ANEXOS

Manuscritos redigidos nas normas do Jornal Veterinary Research

VETERINARY RESEARCH

Title page
The title page should list the title of the article, the full names, institutional addresses, email addresses for all authors. The corresponding author should also be indicated.

Abstract
The Abstract of the manuscript should not exceed 250 words.

Keywords
Three to ten keywords representing the main content of the article.

Introduction
The Introduction section should be written in a way that is accessible to researchers without specialist knowledge in that area and must clearly state - and, if helpful, illustrate - the background to the research and its aims. The section should end with a brief statement of what is being reported in the article.

Materials and methods
The Materials and methods section should include the design of the study, the type of materials involved, a clear description of all comparisons, and the type of analysis used to enable replication.

Results and discussion
The Results and discussion may be combined into a single section or presented separately. The Results and discussion sections may also be broken into subsections with short, informative headings. Conclusions should be indicated in this section.

List of abbreviations
If abbreviations are used in the text they should be defined in the text at first use, and a list of abbreviations can be provided, which should precede the competing interests and authors' contributions.

Competing interests
A competing interest exists when your interpretation of data or presentation of information may be influenced by your personal or financial relationship with other people or organizations. Authors must disclose any financial competing interests; they should also reveal any non-financial competing interests that may cause them embarrassment were they to become public after the publication of the manuscript. Authors are required to complete a declaration of competing interests. All competing interests that are declared will be listed at the end of published articles. Where an author gives no competing interests, the

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