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Natália Hettwer Pedroso

UM PCR/RT-PCR *MULTIPLEX* PARA A DETECÇÃO DE CINCO AGENTES DA DIARREIA NEONATAL BOVINA

Santa Maria, RS
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Orientador: Prof. Dr. Eduardo Furtado Flores
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Aprovado em 7 de fevereiro de 2023:

Eduardo Furtado Flores, PhD (UFSM)
(Presidente/Orientador)

José Valter Joaquim Silva Júnior, Dr (UFSM)
(Coorientador)

Juliana Felipetto Cargnelutti, Drª (UFSM)

Viviani Gomes, Drª (FMVZ-USP)

Rudi Weiblen, PhD (UFSM)
(Suplente)

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RESUMO

UM PCR/RT-PCR MULTIPLEX PARA DETECÇÃO DE CINCO AGENTES DA DIARREIA NEONATAL BOVINA

AUTORA: Natália Hettwer Pedroso
ORIENTADOR: Eduardo Furtado Flores

A diarreia neonatal bovina (DNB) é uma doença complexa que acomete bezerros, especialmente no primeiro mês de vida, e é responsável por altas perdas econômicas, sendo um grande desafio sanitário em rebanhos bovinos de corte e leite. A DNB possui etiologia multifatorial, sendo frequentemente associada a infecções simples ou coinfecções por vírus, bactérias e protozoários. Consequentemente, o diagnóstico laboratorial da DNB geralmente requer testes específicos para cada potencial agente, um processo que pode ser demorado, laborioso, complexo e oneroso. Nesse contexto, descreve-se um PCR/RT-PCR *multiplex* convencional (*end-point*), baseado em *one-step* RT-PCR, para detecção individual ou simultânea dos cinco principais agentes da DNB: rotavírus bovino (BRV), coronavírus bovino (BCoV), *Escherichia coli* K99 (*E. coli* K99), *Salmonella enterica* (*S. enterica*) e *Cryptosporidium parvum* (*C. parvum*). Inicialmente, foram selecionados e/ou desenhados *primers* de alta cobertura para os agentes alvos. Em seguida, padronizou-se o PCR/RT-PCR *multiplex*, otimizando o *mix* (concentração de *primers* e volume de enzima) e as condições do ensaio (desnaturação inicial, temperatura de anelamento e extensão e número de ciclos). Após a otimização da reação, avaliou-se a sensibilidade analítica do *multiplex* para os cinco alvos e, em seguida, avaliou-se o desempenho diagnóstico do ensaio, pelo teste de 95 amostras de fezes diarreicas de bezerros. A especificidade analítica foi avaliada contra outros patógenos de bovinos: vírus da diarreia viral bovina (BVDV), *E. coli* enterotoxigênica (STa) e *Eimeria* spp. O limite de detecção do PCR/RT-PCR *multiplex* foi de 10 unidades infecciosas de BRV, diluição 10^{-2} de um *pool* de amostras positivas para BCoV, 5 x 10^{-4} UFC para *S. enterica*, 5 x 10^{-6} UFC para *E. coli* K99 e 50 oocistos para *C. parvum*. Não foram observadas amplificações inespecíficas de outros potenciais agentes de diarreia bovina. Entre as 95 amostras de fezes analisadas, 50 (52,6%) foram positivas para pelo menos um alvo, sendo 35 infecções por um agente e 15 coinfecções. O BRV foi o agente mais frequente em monoinfecções (16/35), seguido por *Cryptosporidium* spp. (11/35), que também foi o alvo mais comum nas coinfecções (11/15). Todos os resultados positivos e negativos do *multiplex* foram confirmados em reações individuais específicas para cada agente. Dessa forma, desenvolveu-se um PCR/RT-PCR *multiplex* convencional para o diagnóstico mais rápido, fácil e eficiente da DNB, uma estratégia que pode ser útil em futuros estudos clínicos e de vigilância.

Palavras-chave: Diarreia neonatal. Rotavírus bovino. Coronavírus bovino. *Salmonella enterica*. *Escherichia coli* K99. *Cryptosporidium parvum*.

ABSTRACT

AN END-POINT MULTIPLEX PCR/RT-PCR FOR DETECTION OF FIVE AGENTS OF BOVINE NEONATAL DIARRHEA

AUTHOR: Natália Hettwer Pedroso
ADVISER: Eduardo Furtado Flores

Neonatal calf diarrhea (NCD) is a complex disease that affects calves, especially in the first month of life, and is responsible for high economic losses, being a major health challenge in beef and dairy cattle herds. NCD has a multifactorial etiology and is often frequently associated with single or mixed viral, bacterial and protozoal infections. Consequently, laboratory diagnostic of NCD usually requires specific tests for each agent, a time-consuming, laborious, complex and expensive process. Herein, we describe an end-point multiplex PCR/RT-PCR (one-step RT-PCR-based) for individual or simultaneous detection of five major NCD agents: bovine rotavirus (BRV), bovine coronavirus (BCoV), *Escherichia coli* K99 (*E. coli* K99), *Salmonella enterica* (*S. enterica*) and *Cryptosporidium parvum* (*C. parvum*). Initially, we selected and/or designed high-coverage primers. Subsequently, we standardized the multiplex PCR/RT-PCR, optimizing the mix (primer concentration and enzyme volume) and assay conditions (initial denaturation, annealing and extension temperature, and number of cycles). After careful and rigorous optimization, we evaluated the analytical sensitivity of the multiplex for the five targets and then assessed the assay's diagnostic performance by testing 95 clinical samples of diarrheaic calf feces. The analytical specificity was evaluated against other bovine pathogens: bovine viral diarrhea virus (BVDV), *E. coli* heat-stable enterotoxin (STa) and *Eimeria* spp. The detection limit of our multiplex was 10 infectious units of BRV, 10^{-2} dilution of a BCoV positive sample pool, 5 x 10^{-4} CFU for *S. enterica,* 5 x 10^{-6} CFU for *E. coli* K99 and 50 oocysts for *C. parvum*. No non-specific amplification of other potential bovine diarrhea agents was detected in the assay. Out of 95 samples analyzed, 50 (52.6%) were positive for at least one target, being 35 and 15 single and mixed infections, respectively. BRV was the most frequent agent detected in single infections (16/35), followed by *Cryptosporidium* spp. (11/35), which was also the most frequent agent in mixed infections (11/15). Importantly, positive and negative multiplex results were confirmed in individual reactions for each agent. In conclusion, we described an end-point multiplex PCR/RT-PCR for faster, easier and more efficient NCD diagnosis, which may be useful in future clinical and surveillance studies.

Keywords: Calf diarrhea. Bovine rotavirus. Bovine coronavirus. *Salmonella enterica*. *Escherichia coli* K99. *Cryptosporidium parvum*.

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1 INTRODUÇÃO

A diarreia neonatal bovina (DNB) é uma doença complexa, de etiologia multifatorial, que envolve a interação entre o hospedeiro com fatores nutricionais, ambientais e infecciosos (BARRINGTON; GAY; EVERMANN, 2002; BARTELS et al., 2010). A doença caracteriza-se por um quadro agudo de diarreia, provocando desidratação intensa e desequilíbrio ácido-base e eletrolítico em animais jovens, sendo uma das maiores causas de mortalidade em bezerros, especialmente no primeiro mês de vida, e, por isso, é um grande desafio sanitário em rebanhos bovinos de corte e leite (DALL AGNOL et al., 2021; FOSTER; SMITH, 2009; LORENZ; FAGAN; MORE, 2011).

A DNB também leva a perdas econômicas com mão de obra e medicamentos, retardo de desenvolvimento e ganho de peso, bem como consequências a longo prazo, que incluem atraso no desempenho produtivo e reprodutivo (AGHAKESHMIRI et al., 2017; WITTUM et al., 1994). Além disso, a DNB interfere diretamente no bem-estar animal e estimula o uso excessivo de antimicrobianos, impulsionando o desenvolvimento de resistência bacteriana, podendo também comprometer a saúde humana quando envolve agentes infecciosos potencialmente zoonóticos (AFEMA et al., 2019; IZZO et al., 2011).

Diversos agentes têm sido detectados em casos de DNB, como vírus [*bovine rotavirus* (BRV), *bovine coronavirus* (BCoV), *bovine viral diarrhea virus* (BVDV), *bovine torovirus* (BToV), *bovine astrovirus* (BoAstV) e *bovine norovirus* (BNoV)], bactérias (*Escherichia coli*, *Salmonella* spp. e *Clostridium perfringens*) e protozoários (*Cryptosporidium* spp., *Giardia duodenalis* e *Eimeria* spp.) (BRUNAUER; ROCH; CONRADY, 2021; CHO; YOON, 2014; GOMEZ; WEESE, 2017). Entre esses, o BRV, BCoV, *Escherichia coli* K99 (*E. coli* K99), *Cryptosporidium parvum* (*C. parvum*) e *Salmonella enterica* (*S. enterica*) são considerados os principais agentes envolvidos na DNB (BARKLEY et al., 2021; BLANCHARD, 2012; COURA et al., 2015; FOSTER; SMITH, 2009; IZZO et al., 2011; LANZ UHDE et al., 2008; SMITH, 2012). A prevenção da DNB tem se baseado principalmente na utilização de vacinas para BRV, BCoV, *E. coli* e *S. enterica* (MAIER et al., 2022).

Os rotavírus pertencem à família *Sedoreoviridae*, gênero *Rotavirus* (ICTV, 2021). Os vírions possuem um capsídeo proteico de camada tripla, sem envelope e um genoma composto por 11 segmentos de RNA de fita dupla (*double-stranded*, dsRNA), os quais são

traduzidos em seis proteínas estruturais (*virion proteins*, VPs) e seis não estruturais (*non-structural proteins*, NSPs) (ICTV, 2021). Os rotavírus são classificados em nove grupos (A-J), sendo o BRV do grupo A o agente mais frequente associado aos casos de diarreia em bezerros, inclusive no Brasil (ALFIERI et al., 2006). Nesse grupo, o genótipo predominante é o G6P[5]; no entanto, dois genótipos G (G6 e G10) e dois genótipos P (P[5] e P[11]) também estão circulando no país (DA SILVA MEDEIROS et al., 2019).

Alguns estudos sugerem que o BRV apresenta papel primário no desenvolvimento da DNB, devido à sua alta correlação com sinais clínicos e por estar associado a outros patógenos (BARKLEY et al., 2021; BARTELS et al., 2010; CHO et al., 2013; IZZO et al., 2011; LANZ UHDE et al., 2008). O BRV provoca lise de enterócitos, dano nas vilosidades no intestino delgado e também secreta uma enterotoxina denominada NSP4, capaz de provocar alterações osmóticas no lúmen intestinal (FOSTER; SMITH, 2009). Os animais adultos excretam alta carga viral nas fezes e são a principal fonte de infecção para animais recém-nascidos (GELETU; USMAEL; BARI, 2021). A transmissão pelo contato com outros bezerros, ou pela água, alimentos e fômites também é possível, afetando bezerros nas primeiras semanas de vida (DE OLIVEIRA et al., 2012; GELETU; USMAEL; BARI, 2021).

O diagnóstico da rotavirose em bovinos é baseado em testes como imunoabsorção enzimática (*enzyme linked immuno sorbent assay* – ELISA), transcrição reversa e amplificação de DNA (*reverse transcription-polymerase chain reaction* – RT-PCR), imunocromatografia, imunofluorescência, eletroforese em gel de poliacrilamida (*sodium dodecyl-sulfate polyacrylamide gel electrophoresis* – SDS-PAGE), aglutinação em látex e microscopia eletrônica com amostras de fezes ou fragmentos intestinais (BLANCHARD, 2012; CHO; YOON, 2014) .

Os coronavírus infectam uma grande variedade de hospedeiros (HODNIK; JEŽEK; STARIČ, 2020). Em bovinos, o BCoV pode ser detectado em casos de diarreia em bezerros, disenteria de inverno e doenças do trato respiratório (HODNIK; JEŽEK; STARIČ, 2020). O BCoV é um vírus da família *Coronaviridae*, subfamília *Coronavirinae* e gênero *Betacoronavirus* (ICTV, 2021). Os vírions são envelopados, com genoma de RNA de fita simples (*single-stranded*, ssRNA) de sentido positivo (ICTV, 2021).

O BCoV infecta colonócitos e enterócitos das criptas intestinais, o que resulta em diarreia pelo mecanismo de má absorção e má digestão, especialmente em animais com 16 a 30 dias de idade (ARGENZIO, 1985; FOSTER; SMITH, 2009; STIPP et al., 2009). Bovinos adultos servem de reservatórios de infecção para animais mais jovens, por meio da eliminação do vírus pelas fezes (BARRINGTON; GAY; EVERMANN, 2002). Assim como o BRV, o

BCoV é um importante agente da DNB nos rebanhos bovinos (OLIVEIRA FILHO et al., 2007; STIPP et al., 2009; TAKIUCHI et al., 2006). No entanto, o seu papel como agente primário ainda é questionado devido à frequente detecção em fezes de animais saudáveis (GOMEZ; WEESE, 2017). Além disso, a detecção de BCoV também tem sido correlacionada com tratamento anterior com antimicrobianos e/ou em associação com outros enteropatógenos, sugerindo um comportamento oportunista (BARTELS et al., 2010; OLIVEIRA FILHO et al., 2007). A identificação do BCoV em amostras fecais é realizada principalmente por meio de isolamento em cultivo celular, microscopia eletrônica, ELISA, ensaio de hemaglutinação e técnicas moleculares, como RT-PCR (CHO; YOON, 2014).

A *E. coli* é um microrganismo comensal do trato gastrointestinal. No entanto, algumas cepas que expressam fatores de virulência são capazes de provocar diarreia e outras doenças extra-intestinais em humanos e animais, incluindo a *E. coli* produtora de shigatoxina (*shiga toxin-producing Escherichia coli* – STEC), *E. coli* enteropatogênica (*enteropathogenic Escherichia coli* – EPEC), *E. coli* enterotoxigênica (*enterotoxigenic Escherichia coli* – ETEC), *E. coli* enteroinvasiva (*enteroinvasive Escherichia coli* – EIEC) e *E. coli* enteroagregativa (*enteroaggregative Escherichia coli* – EaggEC) (BOUZARI; JAFARI; ASLANI, 2012).

A diarreia por ETEC é comum em bezerros, principalmente quando essas expressam antígenos de colonização ou aderências, como as fímbrias F5 (*E. coli* K99), que permitem a bactéria colonizar o intestino delgado (FOSTER; SMITH, 2009). A *E. coli* K99 adere-se aos receptores do epitélio intestinal de bovinos por meio de suas fímbrias e secreta enterotoxinas que interferem na absorção e provocam hipersecreção de fluídos e eletrólitos no intestino delgado de neonatos (FOSTER; SMITH, 2009). Dentre as enterotoxinas que a ETEC pode produzir, estão as enterotoxinas termolábeis (*labile enterotoxin* – LT) e as termicamente estáveis (*heat-stable enterotoxi*n – STa e STb), sendo a produção de STa mais comum em bezerros com diarreia (NGELEKA et al., 2019).

A *E. coli* K99 está incluída em vacinas aplicadas a fêmeas prenhes, em associação com antígenos de BRV e BCoV, para proteção de bezerros por meio de uma colostragem de qualidade, fato que pode reduzir a incidência da infecção por essa bactéria (BARTELS et al., 2010; KOLENDA; BURDUKIEWICZ; SCHIERACK, 2015). O diagnóstico da colibacilose neonatal é realizado por meio de cultura microbiológica, ELISA e reação em cadeia da polimerase (*polymerase chain reaction* – PCR) para a triagem das fímbrias e toxinas bacterianas (CHO et al., 2014).

A criptosporidiose é uma infecção de alta prevalência em bovinos em todo o mundo, provocando preocupações do ponto de vista econômico e de saúde pública, uma vez que apresenta potencial zoonótico (SANTÍN; TROUT; FAYER, 2008; WEI et al., 2021). As principais espécies desse protozoário que infectam bovinos são *C. parvum*, *C. bovis* e *C. andersoni* (SANTÍN; TROUT; FAYER, 2008). *C. parvum* possui importância na clínica de bezerros neonatos, enquanto que as outras espécies apresentam-se mais associadas à infecção de animais mais velhos (SANTÍN; TROUT; FAYER, 2008). Assim, bezerros infectados com *C. parvum* podem ser a principal fonte para disseminação da infecção zoonótica (ABEYWARDENA et al., 2012). O *C. hominis* também tem sido identificado em amostras fecais de bezerros saudáveis e com diarreia, embora a importância desses achados ainda seja alvo de discussão (ABEYWARDENA et al., 2012; PARK et al., 2006; RAZAKANDRAINIBE et al., 2018; ROBERTSON et al., 2020). De forma geral, a criptosporidiose provoca atrofia vilosa grave, com perda de células epiteliais, levando a uma diarreia por má absorção e má digestão (ARGENZIO, 1985; FOSTER; SMITH, 2009).

A transmissão da criptosporidiose ocorre pela via fecal-oral, principalmente por meio da contaminação de alimentos, água e das instalações (THOMSON et al., 2017). A prevenção e controle da doença são difíceis devido à possibilidade de autoinfecção, alta resistência dos oocistos no ambiente e disponibilidade limitada de produtos farmacêuticos para tratamento (THOMSON et al., 2017). Além disso, a indisponibilidade de vacinas comerciais dificulta a sua prevenção (THOMSON et al., 2017). Para o diagnóstico da criptosporidiose podem ser utilizados métodos moleculares (PCR), ELISA, cromatografia, flotação fecal, imunofluorescência direta ou esfregaços diretos, com coloração (ATWAL et al., 2022; BLANCHARD, 2012; TROTZ-WILLIAMS et al., 2007).

A *Salmonella enterica* também é uma importante causa de diarreia em bezerros, podendo ser detectada em animais com até três meses de idade (COURA et al., 2015; NGELEKA et al., 2019). A *S. enterica* é uma bactéria gram-negativa, não formadora de esporos, anaeróbia facultativa, que apresenta grande importância para a saúde pública e animal, sendo prevalente em um número considerável de propriedades (IZZO et al., 2011). As espécies mais envolvidas em casos de DNB são: *S. enterica* subsp. *enterica* serovar Dublin (*S.* Dublin) e *S. enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium), as quais provocam diarreia aguda, com alto risco de mortalidade em bezerros (BARKLEY et al., 2021; CAFFARENA et al., 2021; NGELEKA et al., 2019). Apesar disso, vários outros serovares já foram isolados de fezes de bezerros com diarreia, como *S. newport*, *S. enteritidis e S. anatum* (CAFFARENA et al., 2021; CUMMINGS et al., 2009; YOUNIS et al., 2009).

A patogênese da infecção por *S. enterica* envolve a hipersecreção e inflamação intestinal e ocorre, principalmente ao redor do primeiro mês de idade (ARGENZIO, 1985; CHO; YOON, 2014). A *S. enterica* infecta enterócitos na mucosa intestinal e tecidos linfóides, sobrevive no interior de macrófagos, e provoca sinais clínicos como diarreia aquosa amarelada, podendo conter muco e sangue (MOHLER; IZZO; HOUSE, 2009). A *S. dublin* é associada a casos de infecções sistêmicas em bezerros e está relacionada com altos índices de morbidade e mortalidade (MOHLER; IZZO; HOUSE, 2009). O diagnóstico de salmonelose baseia-se em uma ou mais etapas de enriquecimento de cultura bacteriana, seguida por PCR, imunocromatografia ou isolamento em meios seletivos (BLANCHARD, 2012).

Na maioria dos casos e surtos de DNB, os sinais clínicos são o resultado da associação entre os agentes citados acima (BRUNAUER; ROCH; CONRADY, 2021; COURA et al., 2015; CHO et al., 2013; DALL AGNOL et al., 2021; IZZO et al., 2011). Embora a detecção de enteropatógenos muitas vezes possa ocorrer em animais saudáveis, coinfecções são mais comuns em animais com diarreia (BRUNAUER; ROCH; CONRADY, 2021). As coinfecções mais frequentes na DNB são: BRV/*Cryptosporidium* spp.*,* seguida da associação entre BRV/BCoV e BRV/*E. coli* K99 (BRUNAUER; ROCH; CONRADY, 2021). A presença de coinfecções na DNB pode ser responsável pela rápida evolução clínica da doença, agravando o quadro agudo de desidratação, desequilíbrio eletrolítico, acidose metabólica e choque hipovolêmico, o que reforça a necessidade de um diagnóstico rápido e preciso, útil para a implementação do tratamento adequado e evitando maiores perdas na criação de bezerros (CHO; YOON, 2014; GOMEZ; WEESE, 2017; NAYLOR, 2009).

Nesse sentido, os testes moleculares, como PCR e RT-PCR, tornam-se atrativos para o diagnóstico laboratorial da DNB, principalmente devido à alta sensibilidade e especificidade, além da rapidez da entrega dos resultados (CHO; YOON, 2014; YOUNIS et al., 2009). Os testes moleculares para DNB também têm sido usados em plataformas *multiplex* baseadas, principalmente, em reações de tempo real/quantitativas (*quantitative* PCR, qPCR), as quais permitem o diagnóstico diferencial, bem com a detecção simultânea de diferentes agentes em uma mesma amostra biológica, representando uma vantagem ao diagnóstico individual da doença, frequentemente realizado na rotina laboratorial (CHO et al., 2010; GOTO et al., 2020; PANSRI et al., 2022).

Os ensaios de qPCR, no entanto, apresentam um custo elevado relacionado à utilização de sondas e equipamentos específicos, sendo uma opção inviável para muitos laboratórios (PANSRI et al., 2022). Ensaios *multiplex* baseados em reações convencionais (*end-point*) de PCR e/ou RT-PCR, por outro lado, representam alternativas interessantes para

conciliar custo laboratorial com a necessidade de triagem e detecção de vários agentes infecciosos, já tendo sido utilizados com sucesso no diagnóstico diferencial de várias doenças de animais (CARGNELUTTI; WEIBLEN; FLORES, 2017; FONSECA et al., 2011; FUKUDA et al., 2012; HAO et al., 2019). Apesar dessas vantagens, as estratégias de *multiplex* convencionais descritas para o diagnóstico laboratorial da DNB ou contemplam um número pequeno de potenciais agentes infecciosos, a exemplo de ensaios *duplex*, ou apresentam dificuldade de interpretação dos resultados quando mais alvos são incluídos (ASANO et a., 2010; ZHU et al., 2011; WISE; LANSING, 2005).

Por fim, diante da importância das coinfecções em DNB e da necessidade de testes que conciliem alta sensibilidade, alta especificidade, praticidade e baixo custo, o objetivo do presente trabalho foi desenvolver um ensaio RT-PCR/PCR *multiplex* convencional para a detecção de um ou mais agentes associados à doença. O uso dessa plataforma poderá contribuir com o diagnóstico diferencial da DNB, auxiliando também para a definição e implementação de medidas de prevenção e controle.

2 ARTIGO

An end-point multiplex PCR/RT-PCR for detection of five agents of bovine neonatal diarrhea

Natália Hettwer Pedroso[1,2]; José Valter Joaquim Silva Júnior[1,3,4]; Alice Silveira Becker [1,2]; Rudi Weiblen[1]; Eduardo Furtado Flores[1*]

[1]Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Rio Grande do Sul, Brazil.

[2]Programa de Pós-graduação em Medicina Veterinária, Universidade Federal de Santa Maria, Rio Grande do Sul, Brazil.

[3]Setor de Virologia, Instituto Keizo Asami, Universidade Federal de Pernambuco, Pernambuco, Brazil.

[4]Departamento de Análises Clínicas, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Rio Grande do Sul, Brazil

*Correspondence to: Eduardo Furtado Flores. E-mail: eduardofurtadoflores@gmail.com. Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Av. Roraima, 1000, Prédio 63A, Camobi, 97105-900, Santa Maria, Rio Grande do Sul, Brazil.

Abstract

Neonatal calf diarrhea (NCD) is a multifactorial disease frequently associated with single or mixed viral, bacterial and/or protozoal infections. Consequently, laboratory diagnostic of NCD usually requires specific tests for each potential agent; a time-consuming, laborious, complex and expensive process. Herein, we describe an end-point multiplex PCR/RT-PCR (one-step RT-PCR-based) for individual or simultaneous detection of five major NCD agents: bovine rotavirus (BRV), bovine coronavirus (BCoV), *Escherichia coli* K99 (*E. coli* K99), *Salmonella enterica* (*S. enterica*) and *Cryptosporidium parvum* (*C. parvum*). Initially, we selected and/or designed high-coverage primers. Subsequently, we standardized the multiplex PCR/RT-PCR, optimizing the mix (primer concentration and enzyme volume) and assay conditions (initial denaturation, annealing and extension temperatures, and number of cycles). After careful and rigorous optimization, we evaluated the analytical sensitivity of the multiplex for the five targets and then assessed the diagnostic performance of the assay by testing 95 clinical samples of diarrheic calf feces. The analytical specificity was evaluated against other bovine pathogens: bovine viral diarrhea virus (BVDV), *E. coli* heat-stable enterotoxin (STa) and *Eimeria* spp. The detection limit of our multiplex was 10 infectious units of BRV, 10^{-2} dilution of a BCoV positive sample pool, 5 x 10^{-4} CFU for *S. enterica,* 5 x 10^{-6} CFU for *E. coli* K99 and 50 oocysts for *C. parvum*. No non-specific amplification of other potential bovine diarrhea agents was detected in the assay. Out of 95 samples analyzed, 50 (52.6%) were positive for at least one target, being 35 single and 15 mixed infections. BRV was the most frequent agent detected in single infections (16/35), followed by *Cryptosporidium* spp. (11/35), which was the most frequent agent in mixed infections (11/15). Importantly, positive and negative multiplex results were confirmed in individual reactions for each agent. In conclusion, we described an end-point multiplex PCR/RT-PCR for faster,

easier and more efficient NCD diagnosis, which may be useful for routine diagnosis and surveillance studies.

Keywords: Calf. Bovine rotavirus. Bovine coronavirus. *Salmonella enterica*. *Escherichia coli* K99. *Cryptosporidium parvum*.

1 Introduction

Neonatal calf diarrhea (NCD) is a complex and multifactorial disease which includes interactions among environmental, nutritional and infectious factors (Barrington et al., 2002; Bartels et al., 2010). Clinically, NCD presents as acute diarrhea, leading to severe dehydration in young animals and being the main cause of death in calves in the first month of life (Caffarena et al., 2021; Dall Agnol et al., 2021). To date, NCD remains a major challenge in cattle herds worldwide, leading to labor and treatment costs, impacts on welfare and production and high mortality, which may reach more 50% of causes of mortality in weaned calves (Aghakeshmiri et al., 2017; Caffarena et al., 2021; USDA, 2018).

Several agents have been associated with NCD, namely bovine rotavirus (BRV), bovine coronavirus (BCoV), enterotoxigenic *Escherichia coli* K99 (*E. coli* K99), *Salmonella enterica* (*S. enterica*) and *Cryptosporidium parvum* (*C. parvum*) (Barkley et al., 2021; Coura et al., 2015; Izzo et al., 2011; Lanz Uhde et al., 2008; Smith, 2012). BRV (family *Sedoreoviridae* and genus *Rotavirus*) is a non-enveloped virus, with a genome of 11 segments of double-stranded RNA (dsRNA) (ICTV, 2021). Group A BRV is considered the main enteropathogenic agent associated with NCD (Barkley et al., 2021). BCoV (family *Coronaviridae* and genus *Betacoronavirus*) is an enveloped virus, with a positive-sense single-stranded RNA genome (ssRNA) (ICTV, 2021), detected in severe diarrhea in calves, winter dysentery and respiratory tract diseases (Gomez and Weese, 2017).

Regarding non-viral NCD agents, diarrhea from enterotoxigenic *E. coli* (ETEC), a gram-negative bacillus, is considered common in calves, especially by ETEC that express colonization/adhesions antigen [*e.g.*, K99 (F5) antigen] or heat-stable enterotoxin (STa) (Ngeleka et al., 2019). *S. enterica* is a gram-negative, non-sporulating, facultative anaerobic bacterium, important for human and animal health (Izzo et al., 2011). Although calves may be

infected with several *Salmonella* serovars, *S. enterica* subsp. *enterica* serovar Dublin (*S.* Dublin) and *S. enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) have been associated with severe cases of life-threatening intestinal damage (Caffarena et al., 2021; Ngeleka et al., 2019). *C. parvum* is frequently detected in NCD and calves infected with this protozoan may be the main source of zoonotic infection, being favored by the risk of autoinfection, environmental resistance of oocysts and lack of effective treatment and vaccine (Bartels et al., 2010; Cho and Yoon, 2014; Thomson et al., 2017).

In most NCD cases or outbreaks, the disease results from the association of different pathogens (Coura et al., 2015; Dall Agnol et al., 2021; Izzo et al., 2011). Nevertheless, investigation of NCD-related agents is often performed individually, using molecular assays, traditional virological diagnostic methods (*e.g.*, electron microscopy and cell culture isolation), microbiological culture (for *S. enterica* and *E. coli* K99), direct microscopic examination and/or stool smear staining for *C. parvum* (Goto et al., 2020). Other assays for the detection of BRV, BCoV, *E. coli* K99, *S. enterica* and *C. parvum* include lateral flow immunochromatography and antigen capture ELISA (Bartels et al., 2010; Cho and Yoon, 2014).

Considering the multietiological nature of NCD, the individual investigation of each potential agent may lead to a delay and/or incomplete diagnosis, making it difficult to implement control or prevention measures (Cho et al., 2010). To circumvent this issue, multiplex assays based on real-time reactions (qPCR) have been developed for simultaneous and differential detection of several NCD agents (Goto et al., 2020; Pansri et al., 2022; Thanthrige-don et al., 2018). Alternatively, some authors have also described end-point duplex or multiplex for NCD diagnosis, which may be useful for laboratories with limited access to qPCR platforms (Asano et al., 2010; Wise and Lansing, 2005; Zhu et al., 2011). Despite the success of these approaches, the end-point multiplex assays described for NCD

cover a limited number of agents, leading to difficult interpretations when more targets are included (Wise and Lansing, 2005).

Considering the above and aiming to contribute to a more efficient and easily accessible NCD diagnosis, we developed an end-point multiplex for detection of five NCD-related agents: BRV, BCoV, *E. coli* (K99), *S. enterica* and *C. parvum*. The assay described here allowed for detection of DNA and RNA targets simultaneously and, therefore, was termed multiplex PCR/RT-PCR.

2 Materials and methods

2.1 Study design

A multiplex PCR/RT-PCR was developed for five NCD agents. Initially, we performed a detailed literature search for high-coverage primers. When high-coverage primers were not available or when their amplicons were similar in size, new primer sets were designed. Subsequently, we conducted a rigorous standardization of the multiplex PCR/RT-PCR, optimizing mix (primer concentration and enzyme volume), assay conditions (initial denaturation, annealing and extension temperatures, and number of cycles) and agarose gel concentration for proper interpretation of results. After optimization, we evaluated the analytical sensitivity and specificity of the multiplex PCR/RT-PCR. Finally, to assess the diagnostic performance of our assay, we subjected 95 samples of diarrheic calf feces to single and multiplex PCR/RT-PCR reactions and compared their results.

2.2 Selection and design of primers

The primers previously described by Makino et al. (1999) and Guy et al. (2003) were used for amplification of *S. enterica* and *C. parvum*, respectively. Amplification of BRV, BCoV and *E. coli* K99 was performed with primers designed in the present study and based on 96 (non-structural protein 5, NSP5, gene), 107 (complete genomes) and 14 (fimbrial protein gene, K99) sequences, respectively, available on GenBank. All primers were analyzed for the possibility of dimer formation (https://idtdna.com/pages/tools/oligoanalyzer) and non-specific annealing [Basic Local Alignment Search Tool (BLAST), https://blast.ncbi.nlm.nih .gov/Blast.cgi]. Details on amplified targets, melting temperature and amplicon size are described in Table 1.

2.3 Positive controls

Isolates/samples previously identified as positive for *S. enterica* (provided by Dr. Juliana Felipetto Cargnelutti, *Universidade Federal de Santa Maria*, UFSM), *C. parvum* (provided by Dr. Fernanda Silveira Flores, UFSM), BCoV (provided by Dr. Paulo Brandão, *Universidade de São Paulo*, USP), *E. coli* K99 (provided by Dr. Rodrigo Otávio Silveira Silva, *Universidade Federal de Minas Gerais*, UFMG) and BRV (from Virology Section, UFSM) were used to optimize the multiplex assay and included as positive controls throughout the study. BVDV-1 (Singer strain) (from Virology Section, UFSM), *E. coli* STa (provided by Dr. Juliana Felipetto Cargnelutti, UFSM) and *Eimeria* spp. (provided by Dr. Fernanda Silveira Flores, UFSM) were used for addressing the analytical specificity of the multiplex PCR/RT-PCR.

2.4 Multiplex PCR/RT-PCR development

Several parameters were optimized in the multiplex PCR/RT-PCR assay. Initially, we optimized a preheating step to denature the BRV dsRNA without impairing the amplification of the other agents. Herein, the template (DNA and/or RNA) of all targets were heated to different temperatures (75 to 95°C, with a 5°C interval) for 5min, quickly chilled on ice for 5 min and then used for reaction evaluation. Next, the multiplex PCR/RT-PCR was optimized for final primer concentration (0.2 μM or 0.4μM), enzyme volume (0.6 or 1μL), annealing temperature (50 to 64°C, with a 2°C interval), extension temperature (60 to 72°C, with a 2°C interval) and cycle number (35 or 40). All reactions, including DNA target assays, were performed with AgPath-ID™ One-Step RT-PCR (Applied Biosystems™). In addition, different concentrations of agarose gel (4, 5 or 6%) were evaluated for the correct interpretation of the amplicons. PCR products were stained with GelRed® (Biotium).

The amplicons obtained during the multiplex assay optimization were submitted to nucleotide sequencing. PCR products were purified with PureLink PCR Purification (Invitrogen™), according to the manufacturer's instructions, and subjected to nucleotide sequencing by the Sanger method, using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™) (ACTGene Análises Moleculares Ltda., Brazil).

2.5 Multiplex PCR/RT-PCR sensitivity and specificity

The multiplex PCR/RT-PCR detection limit was defined in infectious units for BRV (using Rotateq® vaccine), in oocysts for *C. parvum* and in CFU for *S. enterica* and *E. coli* K99. For BCoV, the limit of detection was based on dilution of a pool of positive samples.

The analytical specificity of the multiplex PCR/RT-PCR was evaluated against other agents associated to bovine diarrhea (BVDV, *E. coli* STa and *Eimeria* spp). The suitability of the DNAs/RNAs was assured by their amplification with the primers described by Monteiro

et al. (2019) (BVDV), Bosworth & Casey (1997) (*E. coli* STa) and Malek & Kuraa (2018) (*Eimeria* spp.).

2.6 Diagnostic performance of the multiplex PCR/RT-PCR

Ninety-five clinical samples of diarrheic calf feces received at the Virology Section (UFSM) for diagnostic purposes (2020-2022) were used to evaluate the diagnostic performance of the multiplex PCR/RT-PCR. Samples were stored at -80°C until processing.

Total DNA and RNA were extracted together using an adapted protocol based on TRIzol™ (Invitrogen™). Briefly, after the chloroform treatment step, the DNA phase was collected, washed twice with chloroform:isoamyl alcohol (24:1, v/v) and then pooled with the RNA phase. The following steps were performed according to the manufacturer's protocol. DNA/RNA was eluted in 40µL Tris-EDTA (10mM Tris-HCl, 1mM EDTA, pH 8.0) and the concentration and quality of nucleic acids were determined spectrophotometrically (NanoDrop™ Lite Spectrophotometer, Thermo Scientific™). DNA/RNA was kept at -80°C until their use. Finally, nucleic acids were subjected to single and multiplex reactions to detect all targets, and the positivity and negativity rates of each assay were compared. Conditions for single reactions are detailed in Supplementary File 1.

3 Results

3.1 Optimization of the multiplex PCR/RT-PCR assay

The multiplex assay was developed and standardized using AgPath-ID™ One-Step RT-PCR (Applied Biosystems™). To facilitate the reverse transcription of the BRV dsRNA

without impairing the amplification of the other targets, the templates were initially denatured by heating at 80°C for 5 min and then quickly chilled on ice for 5 min before multiplex PCR/RT-PCR. The reaction was optimized to a final volume of 15µL: 7.5µL of 2X RT-PCR buffer, 1µL of Taq Enzyme Mix, 0.12µL of forward primers (25µM), 0.12µL of reverse primers (25µM), 0.3µL of free nuclease water and 5µL of template (DNA and/or RNA). Reaction conditions were as follows: 45°C for 30min (reverse transcription), 95°C for 10min (initial denaturation and reverse transcriptase inactivation), followed by 35 cycles of denaturation at 95°C for 1min, annealing/entension at 62°C for 1min. PCR products were best resolved on 5 and 6% agarose gels, preferentially choosing the lowest concentration. Here, the five individual targets were easily observed and differentiated: BCoV (323bp), *S. enterica* (260bp), *E. coli* K99 (190bp), BRV (170bp) and *C. parvum* (151bp) (Figure 1A). The identity of the agents was confirmed by nucleotide sequencing of the amplicons (data not shown).

3.2 Multiplex PCR/RT-PCR sensitivity and specificity

The detection limit of the multiplex was 10 infectious units for BRV, 10^{-2} dilution of a BCoV positive sample pool (SV 174/21-201, SV 223/22-1 and SV 223/22-4), 5 x 10^{-4} CFU for *S. enterica,* 5 x 10^{-6} CFU and 50 oocysts for *C. parvum*. Regarding the analytical specificity, none of the other agents involved in bovine diarrhea were amplified by the multiplex assay (Figure 1B).

3.3 Diagnostic performance of the multiplex PCR/RT-PCR

Among the 95 diarrheic stool samples analyzed by the multiplex assay, 52.6% (50/95) were positive for at least one of the investigated targets. Among the single infections, 16 were

positive for BRV, 11 for *Cryptosporidium* spp., 6 for BCoV and 2 for *E. coli* K99 (Table 2). Mixed infections were detected in 15 samples: five were positive for BCoV, *E. coli* K99 and *Cryptosporidium* spp., four for *E. coli* K99 and BRV, three for BRV and *Cryptosporidium* spp., two for *S. enterica* and *Cryptosporidium* spp. and one for BCoV and *Cryptosporidium* spp (Table 2). Details on the results are presented in Supplementary File 2. Importantly, as the primers used for *Cryptosporidium* may also amplify species that infects humans, including *C. hominis* (Guy et al., 2003), positive samples were identified as *Cryptosporidium* spp.

All samples identified as positive in the multiplex assay, including co-infections, were also positive when submitted to individual reactions. Similarly, all samples negative in the multiplex assay were also negative in individual reactions. Non-specific amplifications were not observed in any of the samples analyzed by the multiplex PCR/RT-PCR (data not shown).

4 Discussion

The main obstacles in the etiological diagnosis of NCD include the number of potential agents and the high frequency of co-infections. Consequently, complete NCD investigation usually requires specific tests for each agent, making the laboratory diagnosis a time-consuming, complex and expensive process. In order to contribute to an easier, faster, more efficient and less expensive NCD diagnosis, we developed an end-point multiplex PCR/RT-PCR for single or simultaneous detection of five NCD-related agents: BRV, BCoV, *C. parvum*, *S. enterica* and *E. coli* K99. According to the literature, these are the most important agents involved in NCD worldwide (Barkley et al., 2021; Coura et al., 2015; Izzo et al., 2011; Lanz Uhde et al., 2008; Smith, 2012).

Initially, we selected and/or designed high-coverage primers for the five targets. The primers used for amplification of *S. enterica* and *C. parvum* have been described by Makino

et al. (1999) and Guy et al. (2003), respectively, and have been frequently used for the detection of these agents (Cho et al., 2010; Cho et al., 2013; ElGamal and ELBahi, 2016; Thanthrige-Don et al., 2018; Wang et al., 2023; Ziemer and Steadham, 2003). Primers for BCoV, BRV and *E. coli* K99 were designed for the study and fully annealed in all sequences in our database (see 2.2 section). Overall, the primers used in our multiplex PCR/RT-PCR allowed for simultaneous and differential detection of all five targets on a 5% agarose gel. Furthermore, unspecific amplifications were not observed in our assay, which was probably favored by the high PCR annealing temperature (62°C). These are important findings, especially considering that the multiplex complexity increases as more targets are included in the reaction. Indeed, regarding NCD, to the best of our knowledge, only real-time or microarray-based multiplex have a comparable number of targets to our assay (Cho et al., 2010; Goecke et al., 2021; Goto et al., 2020; Pansri et al., 2020; Thanthrige-Don et al., 2018; Tsuchiaka et al., 2016). This difficulty is well exemplified in the end-point multiplex for BRV, BCoV and C. *parvum* described by Wise and Lansing (2005). The authors suggested that a fourth agent, *E. coli* K99 or heat-stable enterotoxin (STa or STb), could be added to the assay, although it was difficult to differentiate some targets on agarose gels. This problem could be solved by visualizing the reaction in polyacrylamide gels, capillary eletrophoresis or qPCR.

For a more efficient BRV amplification, an initial heating step to denature the dsRNA prior to the RT reaction is recommended (Freeman et al., 2008; Logan et al., 2006; Wise and Lansing, 2005). Thus, an additional obstacle in our study was to amplify the BRV dsRNA without impairing the detection of other agents. Denaturing at higher temperatures improved BRV amplification, however, amplification of other targets (mainly BCoV) was substantially impaired (data not shown). So, placing all targets on the balance, we found that denaturing at

80°C would led to the best result. We hope that these data will contribute for the optimization of future multiplex assays where it is necessary to combine agents with different genomes.

In order to reduce time and cost of laboratory NCD diagnosis, we extracted DNA and RNA in a single protocol. We also used the AgPath-ID™ One-Step RT-PCR Kit for simultaneous amplification of DNA and RNA targets in a single tube. The advantage of joining extraction of DNA and RNA targets for NCD diagnosis has also been recognized by other authors using different strategies and kits (Goto et al., 2020; Wise and Lansing, 2005). Furthermore, amplification of DNA/RNA targets in a single reaction has also been successfully described in other conventional, real-time and microarray-based multiplex (Dong et al., 2022; Thanthrige-Don et al., 2018; Wise and Lansing, 2005).

Importantly, although the kit used in our study is designed for real time reactions, we standardized it for end-point multiplex assay, including optimizing the extension temperature, a parameter often overlooked during PCR optimization. We also optimized the assay to 15µL of final volume, considerably lowering the cost per reaction. The cost reduction is accentuated when we consider that one-step RT-qPCR kits are often more cost-effective than those used in end-point one-step reactions. The choice by AgPath-ID™ One-Step RT-PCR is also supported by its use in several multiplex for both animal and human infectious agents (Bubba et al., 2015; Laamiri et al., 2018; Ma et al., 2015; Wang et al., 2019).

After careful and rigorous optimization, we evaluated the diagnostic performance of our assay testing 95 fecal samples submitted to our diagnostic service. Among these, 50 (52.6%) were positive for at least one agent and 15 (30%) were positive for two or more NCD-related agents. All single or mixed infections, as well as negative results, were confirmed in target-specific reactions, supporting multiplex performance. These findings are in line with the specificity of our assay, which did not amplify other potential NCD agents, such as BVDV, *E. coli* STa and *Eimeria* spp. On the other hand, the high number of negative

samples detected among the analyzed specimens would be somewhat surprising. It is conceivable that some of the negative results may be explained by infection by other agents not included in the study, such as BVDV, bovine norovirus (BNoV), *Clostridium perfringes* or *Eimeria* spp. (Bartels et al., 2010; Enemark et al., 2013; Cho et al., 2013; Dall agnol et al., 2021). Likewise, the presence of one or more targets in amounts below the detection limit of our assay cannot be ruled out to explain some negative results. In addition, calf diarrhea may also be due to non-infectious causes related to animal handling, such as nutritional aspects and stress (Barrington et al., 2002; Bartels et al., 2010; Dall agnol et al., 2021). Finally, the possibility of sample degradation during transport and/or storage must not be ignored.

In our testings, the BRV was the most frequently detected target. This finding is in line with previous reports, which describe BRV (group A) as the most common agent associated with NCD, being suggested as the primary agent of the disease (Al Mawly et al., 2015; Barkley et al., 2021; Bartels et al., 2010). *Cryptosporidium* spp. was the second most frequent agent identified in our study. Previous studies also have reported an increase in *Cryptosporidium* spp. detection related to the use of more sensitive diagnostic methods (Brunauer et al., 2021; Cho et al., 2013). In addition, *Cryptosporidium* spp. was the most common agent detected in co-infections in our study, mainly with *E. coli* K99 and BCoV. Co-detection of *Cryptosporidium* spp. and other agents has also been reported by other authors and illustrates the multi-causal nature of NCD (Brunauer et al., 2021; De La Fuente et al., 1999; Lanz Uhde et al., 2008; Mohteshamuddin et al., 2020).

Six samples were positive only for BCoV whereas other six showed co-infections: five with *E. coli* K99 and *Cryptosporidium* spp. and one with *Cryptosporidium* spp. The finding of BCoV in mixed infections is in agreement with previous studies (Cho et al., 2013; Coura et al., 2015; Oliveira Filho et al., 2007). BCoV has been frequently detected in association with

BRV and *E. coli* K99, which contributes to the discussion about its questionable role as a primary agent of NCD (Bartels et al., 2010; Brunauer et al., 2021; Lanz Uhde et al., 2008).

Single *E. coli* K99 infection was detected in two samples. On the other hand, we detected *E. coli* K99 in nine co-infected samples with BCoV and *Cryptosporidium* spp. or BRV. Although *E. coli* K99 affects calves mainly in the first week of life, it may also be reported in older animals, often in association with other agents, such as BCoV and BRV (Bartels et al., 2010; Brunauer et al., 2021; Lanz Uhde et al., 2008). Unfortunately, most samples included here lacked data on animal age, but this is an interesting issue and deserves further investigation.

Regarding *Salmonella* spp., its detection has been described only sporadically, but frequently in association with *Cryptosporidium* spp. or BRV (Al Mawly et al., 2015; Cho et al., 2013; Mohtshamuddin et al., 2020). Our findings are in agreement with previous reports and *S. enterica* was detected in only two samples, both co-infected with *Cryptosporidium* spp. Despite the low detection rate, it is important to consider the diagnosis of *Salmonella* spp. since it is related to a high risk of severe disease, pre-weaning mortality and zoonotic infection (Oliveira et al., 2007; Caffarena et al., 2021; Izzo et al., 2011).

In conclusion, we developed an end-point multiplex PCR/RT-PCR assay for differential and simultaneous detection of BRV, BCoV, *E. coli* K99, *S. enterica* and *C. parvum*; the most important agents of NCD. Overall, our assay showed equivalent performance to specific single-target reactions and non-specific amplifications were not observed. In this context, we believe that the multiplex described here represents a considerable cost-effective tool, being an option to improve laboratory diagnosis and surveillance of NCD.

Glossary

BCoV	Bovine coronavirus
BNoV	Bovine norovirus
BRV	Bovine rotavirus
BToV	Bovine torovirus
BVDV	Bovine viral diarrhea virus
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
C. parvum	*Cryptosporidium parvum*
C. hominis	*Cryptosporidium hominis*
E. coli	*Escherichia coli*
ICTV	International Committee on Taxonomy of Viruses
NCD	Neonatal calf diarrhea
S. enterica	*Salmonella enterica*
SV	Virology Section
UFSM	Universidade Federal de Santa Maria

Declarations

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Conflicts of interest/Competing interests

The authors declare that they have no competing interests.

Ethics approval

Not applicable. The study did not involve animal experimentation. The samples used in the study came from the diagnostic service offered by Virology Section (UFSM).

Authors' contributions

NHP: Conceptualization, Methodology, Investigation, Writing – Original draft preparation. JVJSJr: Conceptualization, Methodology, Investigation, Visualization, Writing – Review & Editing. ASB: Methodology, Investigation, Visualization. RW: Conceptualization, Visualization, Writing – Review & Editing. EFF: Conceptualization, Methodology, Visualization, Writing – Review & Editing, Supervision.

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Figure and caption

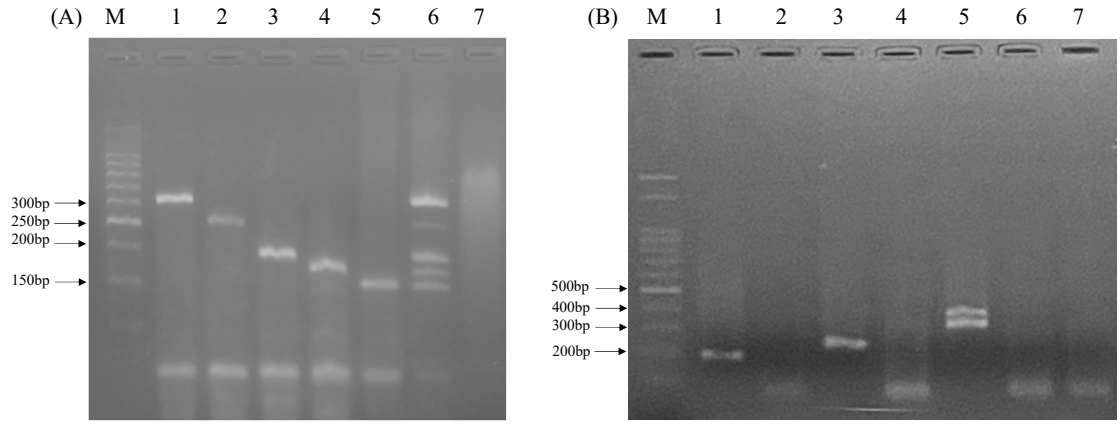

Figure 1: Multiplex PCR/RT-PCR performance. (A) Individual and simultaneous amplification of the five multiplex targets (positive controls) during assay optimization. M: 50bp DNA Ladder (marker) (Ludwig, RS, Brazil); 1: bovine coronavirus (323pb); 2: *Salmonella enterica* (260bp); 3: *Escherichia coli* K99 (190bp); 4: bovine rotavirus (170bp); 5: *Cryptosporidium parvum* (151bp); 6: multiplex PCR/RT-PCR with simultaneous amplification of the five targets; 7: negative control (multiplex reaction with negative sample). (B) Multiplex analytical specific test against *E. coli* STa, bovine viral diarrhea virus (BVDV) and *Eimeria* spp. The quality of *E. coli* STa, BVDV and *Eimeria* spp. DNAs/RNAs was evaluated with target-specific primers, generating 158bp (1), 201bp (3) and 348-546bp (5) amplicons, respectively [Different *Eimeria* spp. Amplicons are related to the presence of different species (Kawahara et al., 2010)]. 2, 4 and 6: multiplex reactions with *E. coli* STa, BVDV and *Eimeria* spp., respectively; 7: negative control (multiplex reaction with water); M: 100bp DNA Ladder (marker) (Ludwig, RS, Brazil).

Table 1: Primer sets used in the end-point multiplex PCR/RT-PCR

Primer	Sequence (5'-3')	Melting temperature (°C)[a]	Nucleic acid	Agent	Amplicon (bp)	Reference
COWP P702-F	CAAATTGATACCGTTTGTCCTTCTG	54.5	dsDNA[b]	*C. parvum* (COWP)[e]	151	Guy et al. (2003)
COWP P702-R	GGCATGTCGATTCTAATTCAGCT	55.3				
BRV-NSP5-F2	CTTTCTGGAAAATCTATTGGTAGGAG	53.3	dsRNA[c]	Bovine rotavirus (NSP5)[f]	170	In this study
BRV-NSP5-R2	GCATTTGTCTTAACTGCATTCG	52.9				
E.coli-K99-O55 strain-196F	GACTGGTCTGGTTCTATGAAC	52.6	dsDNA	*E. coli* K99 (K99)[g]	190	In this study
E.coli-K99-O55 strain-385R	CTGAATAGTTAAATGACTGAATTGC	50.7				
STN-101	CTTTGGTCGTAAAATAAGGCG	52	dsDNA	*S. enterica* (STN)[h]	260	Makino et al., (1999)
STN-111	TGCCCAAAGCAGAGAGATTC	55.1				
BECoV-1a-F3	TTGTGGTGCTTAATGCCCAC	56.1	ssRNA[d]	Bovine coronavirus (ORF1a)[i]	323	In this study
BECoV-1a-R3	CCATCAGCAGAAAGTGCATGTG	56.8				

[a] Calculated at https://www.idtdna.com/calc/analyzer (for 50 mM monovalent salt, Na^+);

[b] double-stranded DNA;

[c] double-stranded RNA;

[d] single-stranded RNA;

[e] *Cryptosporidium* oocyst wall protein;

[f] Nonstructural protein gene 5;

[g] Fimbrial protein gene;

[h] *Salmonella* enterotoxin gene;

[i] Open reading frame one.

Table 2: Positive samples detected by the multiplex PCR/RT-PCR.

Infections	n[a]	BRV[b]	*Cryptosporidium* spp.	BCoV[c]	*E. coli* K99[d]	*S. enterica*[e]
Single	35	16	11	6	2	-
Double	4	+			+	
	3	+	+			
	2		+			+
	1		+	+		
Triple	5		+	+	+	

[a] Number of positive samples;

[b] Bovine rotavirus;

[c] Bovine coronavirus;

[d] *Escherichia coli* K99;

[e] *Salmonella enterica.*

Supplementary File 1: Single PCR and RT-PCR reactions

Individual RT-PCR reactions for BCoV and BRV were standardized by two-step methodology, with cDNA synthesized using the GoScript™ Reverse Transcription Mix Kit, Random Primers (Promega), following the manufacturer's recommendations. Then PCR was performed using the Taq DNA Polymerase, Recombinant (Thermo Fisher Scientific) to a final volume of 25µL: 2µL of cDNA, 2.5µL of each primer (both at 10µM), 2.5µL of $MgCl_2$ (50mM), 2.5µL of dNTPs (10mM), 2.5µL of buffer (10X) and 0.2µL of Taq DNA Polymerase (5U/µL) and 12.8µL of nuclease-free water. Both reactions were carried out under the following conditions: initial denaturation (94°C for 3min), 35 cycles of 94°C for 45sec, annealing at 54°C for 45sec (BRV)/ 58°C for 45sec (BCoV) and extension at 72°C for 30sec, followed by a final extension at 72°C for 7min.

Individual PCR reactions for DNA target (*Salmonella enterica*, *Cryptosporidium parvum* and *Escherichia coli* K99) were performed with the Taq DNA Polymerase kit, Recombinant (Thermo Fisher Scientific); all with final volume of 25µL. The PCR conditions for *E. coli* K99 were 94°C for 3 min (initial denaturation), 35 cycles of denaturation at 94°C for 45sec, annealing at 54°C for 30sec, 72°C for 30sec (extension) and 72°C for 10 min (final extension). The reaction was performed with 2µL of DNA, 2.5µL of each primer (both at 10µM), 1µL of $MgCl_2$ (50mM), 2.5µL of dNTPs (0.2mM), 2.5µL of buffer (10X), 0.2µL of Taq DNA Polymerase (5U/µL) and 14.3µL of nuclease-free water.

For *S. enterica*, the reaction was performed with 2µL of DNA, 2.5µL of each primer (both at 10µM), 1µL of $MgCl_2$ (50mM), 2.5µL of dNTPs (0.2mM), 2.5µL of buffer (10X), 0.2µL of Taq DNA Polymerase (5U/µL) and 14.3µL of nuclease-free water. The reaction conditions were: initial denaturation (94°C for 3min), 35 cycles of 94°C for 1min, annealing at 55°C for 1min and extension at 72°C for 1min, followed by a final extension at 72°C for 10min.

For *C. parvum*, the reaction was performed with 2µL of DNA, 2.5µL of each primer (both at 10µM), 1µL of $MgCl_2$ (50mM), 2.5µL of dNTPs (0.2mM), 2.5µL of buffer (10X), 0.2µL of Taq DNA Polymerase (5U/µL) and 14.3µL of nuclease-free water. The reaction conditions were: initial denaturation (94°C for 3min), 35 cycles of 94°C for 45sec, annealing at 58°C for 45sec and extension at 72°C for 30sec, followed by a final extension at 72°C for 10min.

Details of samples tested by PCR/RT-PCR multiplex

Sample	BRV[a]	BCoV[b]	*Cryptosporidium* spp.	*E. coli* K99[c]	*S. enterica*[d]
SV 204/20[e]	+	-	-	+	-
SV 03/21	-	-	-	-	-
SV 70/21 - 11	-	-	+	-	+
SV 70/21 - 13	-	-	+	-	-
SV 70/21 - 15	-	-	-	-	-
SV 70/21 - 69	+	-	+	-	-
SV 70/21 - 70	+	-	-	-	-
SV 70/21 - 72	-	-	-	-	-
SV 70/21 - 77	+	-	-	-	-
SV 70/21 - 78	-	-	-	-	-
SV 70/21 - 80	-	-	+	-	-
SV 70/21 - 81	+	-	-	-	-
SV 70/21 - 82	+	-	-	-	-
SV 70/21 - 89	+	-	-	+	-
SV 114/21	-	-	-	-	-
SV 159/21 - 2113	+	-	-	-	-
SV 159/21 - 2114	+	-	-	-	-
SV 159/21 - 2117	+	-	-	-	-
SV 167/21	-	-	+	-	+
SV 174/21 140	-	-	-	-	-
SV 174/21 201	-	+	-	-	-
SV 174/21 152	-	-	-	-	-
SV 223/21 - 1	-	+	-	-	-
SV 223/21 - 2	-	-	-	-	-
SV 223/21 - 3	-	-	-	-	-
SV 223/21 - 4	-	+	-	-	-
SV 223/21 - 5	-	+	+	+	-
SV 223/21 - 6	-	+	+	+	-
SV 223/21 - 7	-	+	+	+	-
SV 223/21 - 8	-	+	+	+	-
SV 223/21 - 9	-	+	+	+	-
SV 233/21 - 1832	-	-	+	-	-
SV 233/21 - 2362	-	-	-	-	-
SV 233/21 - 3170	+	-	-	+	-
SV 233/21 - 3141	-	-	-	-	-
SV 233/21 - 3148	-	-	-	-	-
SV 233/21 - 3331	-	-	+	-	-
SV 233/21 - 3332	-	-	+	-	-
SV 233/21 - 3333	-	-	-	-	-

SV 233/21 - 3149	-	-	-	-	-
SV 233/21 - 3150	-	-	-	-	-
SV 233/21 - 3164	-	-	-	-	-
SV 238/21	-	-	-	-	-
SV 239/21	+	-	-	-	-
SV 321/21 - 1	-	-	-	-	-
SV 321/21 - 1	-	-	-	-	-
SV 321/21 - 3	-	-	-	-	-
SV 322/21 - 1	+	-	-	-	-
SV 322/21 - 2	-	-	-	-	-
SV 322/21 - 3	-	-	-	-	-
SV 322/21 - 4	-	-	+	-	-
SV 322/21 - 5	-	-	+	-	-
SV 322/21 - 6	-	-	-	-	-
SV 326/21 - 1	-	+	+	-	-
SV 326/21 - 2	-	+	-	-	-
SV 327/21	-	-	-	-	-
SV 330/21	-	-	+	-	-
SV 333/21	-	-	-	-	-
SV 342/21	-	-	-	-	-
SV 347/21 - 1	+	-	-	-	-
SV 347/21 - 2	-	-	+	-	-
SV 347/21 - 3	-	-	-	-	-
SV 359/21 - 1	+	-	-	-	-
SV 359/21 - 2	+	-	-	-	-
SV 362/21 - 1	-	+	-	-	-
SV 362/21 - 2	-	+	-	-	-
SV 363/21	-	-	-	-	-
SV 367/21	-	-	+	-	-
SV 07/22 - 1	-	-	-	-	-
SV 07/22 - 1	-	-	-	-	-
SV 07/22 - 3	+	-	+	-	-
SV 07/22 - 4	-	-	-	-	-
SV 21/22 - 1	-	-	-	-	-
SV 21/22 - 2	+	-	-	-	-
SV 21/22 - 3	-	-	-	-	-
SV 21/22 - 4	-	-	-	-	-
SV 21/22 - 5	+	-	-	-	-
SV 21/22 - 6	-	-	-	-	-
SV 21/22 - 7	-	-	-	-	-
SV 21/22 - 8	-	-	-	-	-
SV 21/22 - 9	-	-	-	-	-
SV 21/22 - 10	-	-	-	-	-

SV 72/22 - 27	+	-	-	-	-
SV 72/22 - 279	-	-	-	-	-
SV 96/22 - 1	-	-	+	-	-
SV 96/22 - 2	+	-	-	+	-
SV 96/22 - 3	-	-	-	-	-
SV 164/22	-	-	-	+	-
SV 165/22	-	-	-	-	-
SV 166/22	+	-	-	-	-
SV 178/22 - 1	-	-	-	-	-
SV 178/22 - 2	-	-	-	-	-
SV 240/22	+	-	+	-	-
SV 241/22	-	-	+	-	-
SV 319/22	-	-	-	+	-

[a] Bovine rotavirus coronavirus;

[b] Bovine coronavirus;

[c] *Escherichia coli* K99;

[d] *Salmonella enterica*;

[e] SV: protocol record of the Virology Section (*Universidade Federal de Santa Maria*, UFSM)

CONCLUSÃO

Foi desenvolvido um ensaio de PCR/RT-PCR *multiplex* convencional (*end-point*) para detecção diferencial e simultânea dos cinco principais agentes envolvidos na DNB: BRV, BCoV, *E. coli* K99, *S. enterica* e *C. parvum*. No geral, o ensaio mostrou desempenho equivalente às reações individuais e não foram observadas amplificações inespecíficas. Nesse contexto, acredita-se que o *multiplex* aqui descrito representa uma ferramenta de considerável custo-benefício, sendo uma opção para incrementar o diagnóstico laboratorial e a vigilância da DNB.

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