HIGOR SETTE PEREIRA

DESENVOLVIMENTO DE MOLÉCULAS APLICADAS NO DIAGNÓSTICO SOROLÓGICO E MOLECULAR DE DOENÇAS BOVINAS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Bioquímica Aplicada, para obtenção do título de *Doctor Scientiae*.

Orientador: Tiago Antônio de Oliveira Mendes

Coorientadora: Jiehua Zhou

VIÇOSA - MINAS GERAIS
2021

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T

Pereira, Higor Sette, 1992-

P436
2021

Desenvolvimento de moléculas aplicadas no diagnóstico sorológico e molecular de doenças bovinas / Higor Sette Pereira. – Viçosa, MG, 2021.

123 f. : il. (algumas color.) ; 29 cm.

Inclui anexos.
Orientador: Tiago Antonio de Oliveira Mendes.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Técnicas de visualização da superfície celular. 2. Técnica de seleção de aptâmeros. 3. Proteínas quiméricas. 4. Triagem genética - Simulação por computador. I. Universidade Federal de Viçosa. Departamento de Bioquímica e Biologia Molecular. Programa de Pós-Graduação em Bioquímica Aplicada. II. Título.

CDD 22. ed. 572.8

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APROVADA: 1 de abril de 2021.

Assentimento:

Higor Sette Pereira
Autor

Tiago Antônio de Oliveira Mendes
Orientador

AGRADECIMENTOS

Na minha caminhada até aqui, nunca estive só.

À minha esposa, Ana Paula, e minha filha, Isabel, por todo amor, carinho e companheirismo. Esta tese não seria possível sem vocês do meu lado. Saibam que essa vitória é por vocês e para vocês. Amo incondicionalmente vocês duas!

Aos meus familiares, pai, mãe, avó, avô (*in memorian*), irmão, tias e primas, por todo apoio, amor e por acreditarem em mim. A todos os familiares da Ana Paula também, meu mais sincero carinho por toda a confiança e carinho. Amo todos vocês!

Ao professor Tiago Mendes e à professora Andréa Ribon, por toda orientação, ensinamentos e discussões. Essa jornada até aqui só foi possível também por que tive excelentes orientadores que acreditavam em mim. Serei eternamente grato por tudo, muito obrigado!

Aos amigos do LBM por tornarem o dia a dia no laboratório agradável e divertido, com as melhores piadas e lanches. Em especial, ao meu amigo Renato, por ser amigo de longa data, compartilhar comigo todas as aflições e ser, para mim, uma grande referência. Aos demais amigos, Paty, Fernanda, Lílian, Ananda, Ramila, Thays, Igor, Luana, Duda, Lis e vários outros que compartilharam bancada comigo, meu muito obrigado!

Ao Eduardo por tornar todas as etapas burocráticas mais simples, além de ser um grande amigo e vizinho. Muito obrigado por todo acolhimento e ajuda. Você é especial para nós. Isabel sentirá muito sua falta!

Aos amigos de Ponte Nova, que mesmo distantes durante estes tempos difíceis de pandemia, seguem me apoiando e arrancando boas risadas. Vocês são parte fundamental na minha caminhada até aqui.

Agradeço ao Conselho Nacional de Desenvolvimento Técnico e Científico (CNPq) pela concessão da bolsa de estudos. O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

RESUMO

PEREIRA, Higor Sette, D.Sc., Universidade Federal de Viçosa, abril de 2021. **Desenvolvimento de moléculas aplicadas no diagnóstico sorológico e molecular de doenças bovinas.** Orientador: Tiago Antônio de Oliveira Mendes. Coorientadora: Jiehua Zhou.

Falhas reprodutivas que afetam animais de produção geralmente são causadas por agentes infecciosos e geram enormes prejuízos aos produtores. Entre estes patógenos estão os protozoários da tripanossomíase e neosporose bovina. As principais técnicas de diagnóstico se baseiam na percepção dos sinais clínicos, que são inespecíficos. Logo, buscamos estabelecer metodologias eficientes e de baixo custo para desenvolvimento de moléculas que possam ser aplicadas em plataformas de diagnóstico destas duas protozooses. Primeiro, utilizamos da tecnologia do phage-display para rastrear epítopos que diferenciassem soros de animais tripanossomíase positivo daqueles coletados de animais saudáveis, a fim de selecionar uma proteína de *Trypanosoma vivax* para uso no diagnóstico sorológico da infecção. As análises sorológicas indicaram sensibilidade de 88,9% e especificidade de 89,3%, no diagnóstico da tripanossomíase. No segundo capítulo, foi aplicado um rastreio computacional no desenho de uma proteína quimérica para o diagnóstico da neosporose. As análises dos testes sorológicos indicaram sensibilidade de 96,6% e especificidade de 97% na diferenciação dos soros. Na terceira parte, foi estabelecida uma metodologia para seleção de aptâmeros específicos para proteínas-alvo, através de uma metodologia de baixo custo, aplicada para prospecção de aptâmeros que detectem diretamente o patógeno. Um total de cinco sondas de oligonucleotídeos foram identificadas pelo método de Evolução Sistemática de Ligantes por Enriquecimento Exponencial (SELEX) aplicado a alvos proteicos. As evidências experimentais encontradas sugerem que as três metodologias são promissoras e permitem a triagem de novos testes para o diagnóstico de baixo custo, otimizados para uma detecção Point-of-care (POC).

Palavras-chave: Phage-display. Aptâmero. Proteína quimérica. Triagem computacional

ABSTRACT

PEREIRA, Higor Sette, D.Sc., Universidade Federal de Viçosa, April, 2021. **Development of molecules applied in the serological and molecular diagnosis of bovine diseases.** Adviser: Tiago Antônio de Oliveira Mendes. Co-adviser: Jiehua Zhou.

Reproductive failures that affect farm animals are usually caused by infectious agents and generate huge losses for producers. Among these pathogens are trypanosomiasis and neosporosis bovine protozoan. The main diagnostic techniques are based on the perception of nonspecific clinical signs. Therefore, we seek to establish efficient and low-cost methodologies for the development of molecules that can be applied in diagnostic platforms for these two protozooses. First, we used the phage-display technology to screen epitopes that differentiate sera from positive trypanosomiasis animals from those collected from healthy animals, to select a *Trypanosoma vivax* protein for use in the serological diagnosis of the infection. Serological analyzes indicated a sensitivity of 88.9% and specificity of 89.3% in the diagnosis of trypanosomiasis. In the second chapter, a computational screening was applied in the design of a chimeric protein for the diagnosis of neosporosis. The analyzes of the serological tests indicated a sensitivity of 96.6% and specificity of 97% in the differentiation of sera. In the third part, a methodology for the selection of specific aptamers for target proteins was established, through a low-cost methodology, applied prospecting aptamers that will directly detect the pathogen. A total of five oligonucleotide probes were identified by the method of Systematic Evolution of Ligands by EXponential enrichment (SELEX) applied to protein targets. The experimental evidence found suggests that the three methodologies are promising and allow the screening of new tests for low-cost diagnosis, optimized for a Point-of-care (POC) detection.

Keywords: Phage-display. Aptamer. Chimeric protein. Computational screening.

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INTRODUÇÃO GERAL

O uso de terapias ou outros métodos efetivos de controle são dependentes de mecanismos eficientes, práticos e acurados que detectem os patógenos e/ou seus componentes em tempo hábil para aplicação do tratamento correto, seja em doenças humanas ou veterinárias. O requisito fundamental para o tratamento ou controle de uma doença é o diagnóstico precoce, pois aumenta as chances de sobrevivência do indivíduo afetado além de contribuir para a caracterização fisiopatológica dos agentes infecciosos [1]. Na medicina humana, o diagnóstico auxilia na erradicação, tratamento e controle de uma doença em determinada população. Por outro lado, na medicina veterinária, a confirmação de uma doença diminui a necessidade de cirurgias exploratórias e pode também levar o animal ao sacrifício ou abate de maneira a evitar a propagação e potenciais prejuízos em um rebanho, por exemplo. Entretanto, de acordo com o *Committee on Diagnostic Error in Health Care*, acredita-se que todos experimentarão ao menos um erro de diagnóstico ao longo de sua vida, podendo levar a consequências devastadoras em sua saúde [2]. Muitas técnicas de diagnóstico são lentas, custosas e não são capazes de controlar patógenos emergentes com resultados prontamente adaptáveis as novas classes de patógenos [3].

Os pilares para um diagnóstico preciso dos agentes infecciosos estão alicerçados na detecção direta dos patógenos ou de seus componentes, ou na determinação da infecção a partir da detecção de anticorpos específicos contra o patógeno, denominado de diagnóstico sorológico [4]. Os testes sorológicos auxiliam no acompanhamento e identificação dos anticorpos IgG e IgM nas fases agudas em poucos dias após o início da infecção, além de fornecer um panorama geral das infecções que acometeram o indivíduo previamente [5]. Dentre as principais técnicas aplicadas no diagnóstico imunológico, destaca-se o ELISA (do inglês, *Enzyme-Linked ImmunoSorbent Assay*) e o Teste de Imunofluorescência Indireta que fornecem uma plataforma de fácil automação a baixo custo para a avaliação de um grande número de amostras [4]. Nestes ensaios, a aplicação de antígenos recombinantes facilita a padronização, além de possibilitar testes em larga escala contra múltiplos patógenos em um ambiente laboratorial [6].

Por outro lado, as discussões acerca das técnicas moleculares de diagnóstico podem ser divididas em dois períodos: antes e após a era genômica. Antes, esta abordagem molecular era basicamente executada pela detecção do DNA dos microrganismos a partir da Reação em Cadeia da Polimerase (PCR) [7]. Atualmente, neste período pós genômica, ferramentas de alta eficiência capazes de detectar alterações em um único sítio de uma cadeia peptídica ou nucleotídica estão disponíveis para a marcação de organismos e seus componentes, auxiliando na triagem de alvos para o diagnóstico. Nesta pandemia causada pelo vírus SARS-CoV-2 e suas variantes, em uma questão de semanas após o início do surto, uma metodologia para o diagnóstico molecular já havia sido desenvolvida [8]. Além do mais, novas abordagens moleculares foram sendo elucidadas e aprimoradas para a detecção não só dos nucleotídeos DNA e RNA, como também de marcadores contidos no proteoma dos organismos patogênicos. O diagnóstico a partir de abordagens proteômicas, como o MALDI-TOF, é capaz de identificar manifestações fenotípicas de biomarcadores responsáveis ou associados ao processo infeccioso [9].

REVISÃO BIBLIOGRÁFICA

Os bovinos são os animais mais comuns entre os ruminantes. São criados em grandes fazendas com o intuito de produzir leite e carne. Além disso, desempenham um importante papel na ciclagem de nutrientes e na conversão de matéria vegetal não consumível em proteína. No Brasil, a bovinocultura de corte e de leite têm importante papel econômico tanto para o abastecimento do mercado interno quanto para captação de recursos financeiros através da exportação. O Centro de Inteligência da Carne Bovina (CiCarne) é um órgão pertencente à Embrapa Gado de Corte que busca monitorar a cadeia produtiva brasileira para detectar tendências e informações para os produtores dos serviços públicos e privados. Em maio de 2020, mesmo diante de um dos piores cenários econômicos mundiais ocasionados pela pandemia de COVID-19, dados do Ministério da Economia, utilizados pela CiCarne, mostraram que houve acréscimo de 650 mil toneladas na exportação diária de carne bovina - totalizando 6.031 milhões de toneladas ao dia [10]. Não obstante, ao longo de 2021 há ainda uma perspectiva de recuperação do consumo da carne bovina pelo mercado interno, além da aceleração na busca por novos mercados para exportação, além dos contratos já consolidados com países asiáticos como China e Hong Kong [11].

Dada a relevância econômica e alimentícia destes animais em terras brasileiras, muito se investe na manutenção de rebanhos sadios e altamente produtivos. Do ponto de vista econômico, as doenças infecciosas representam um importante desafio, sobretudo pela alta taxa de disseminação e os impactos financeiros gerados nos subprodutos da bovinocultura. Por isso, atenção especial é direcionada para doenças que acometem o trato reprodutivo, uma vez que a rentabilidade do negócio depende da eficiência reprodutiva. Além de afetar diretamente o animal e o rebanho, as doenças reprodutivas podem ser transmitidas verticalmente, afetando ou impedindo o nascimento de novos fetos. Acredita-se que 50% das falhas reprodutivas estejam relacionadas a alguma doença infecciosa [12], porém, esta manifestação clínica por si só não dá indícios conclusivos sobre qual microrganismo afetou o animal. De acordo com alguns estudos [13, 14], as principais doenças infecciosas reprodutivas que acometem os rebanhos brasileiros, são: i) rinotraqueíte infecciosa bovina, causada pelo *Alphaherpesvirus* 1 (BoHV-1); ii) diarreia viral bovina; iii) leptospirose, causada principalmente pela *Leptospira hardjo*; iv) vulvovaginites, causada pelos microrganismos *Mycoplasma bovigenitalium* e *Ureaplasma diversum*; e v) campilobacteriose, causada pela bactéria *Campylobacter fetus*. Além destas causadas por bactérias e vírus, existem protozooses de alta incidência no Brasil que afligem o trato reprodutivo animal. Relatos recentes detectaram problemas reprodutivos associados a infecção causada pelo protozoário *Tritrichomonas foetus*, causador da tricomoníase, nos estados da Paraíba [15] e em Pernambuco [16]. A babesiose, doença que pode ser causada tanto pelo protozoário *Babesia bovis* quanto por *Babesia bigemina*, afeta os animais em regiões subtropicais levando a falhas reprodutivas e também a diminuição da produtividade de leite e carne [17]. No Brasil, testes moleculares e sorológicos indicaram a presença deste parasito em animais no pantanal [17], no noroeste do estado de Minas Gerais [18], na Bahia [19], no Rio de Janeiro [20], em São Paulo [21], Santa Catarina [22] e no Rio Grande do Sul [23]. Entretanto, os principais protozoários causadores de doenças reprodutivas e abortos nos ruminantes são *Neospora caninum* e *Trypanosoma vivax* [24, 25]

Neosporose bovina é uma doença causada pelo protozoário do filo Apicomplexa *Neospora caninum* que, por sua vez, é um parasita intracelular obrigatório comumente associado a problemas reprodutivos em animais em todo o mundo [26]. De acordo com o NCBI, *Neospora caninum* é um organismo celular pertencente ao reino *Eukarya*, clado *Alveolata*, classe *Conoidasida*, ordem *Eucoccidiorida* e família *Sarcocystidae* (NCBI:txid29176). Desde o primeiro relato em cães noruegueses [27], a neosporose tornou-se uma grande preocupação em bovinos de corte e leite [28]. Um estudo de revisão, realizado em 2007, detectou em amostras de soros de bovinos contendo anticorpos anti-*Neospora caninum* em pelo menos 35 países espalhados em todos os continentes [29]. No Brasil, há relatos de infecção em bovinos em praticamente todo o território nacional [30-35]. O aborto é a principal manifestação clínica da doença e pode ocorrer de forma esporádica, endêmica ou epidêmica, entre o terceiro e o nono mês de gestação [24]. No entanto, determinar quais aspectos da infecção estão associados às causas do aborto ainda é um trabalho árduo [36]. Os bezerros sobreviventes mantêm o agente infeccioso em um rebanho, sendo elementos determinantes na epidemiologia da doença. Esses animais são clinicamente saudáveis, soropositivos e escondem o parasita encistado em vários tecidos. A presença contínua de cães nas fazendas está diretamente relacionada ao aumento das infecções por neosporose bovina, uma vez que estes animais são importantes hospedeiros que atuam na transmissão horizontal ao rebanho [37].

A transmissão da neosporose bovina ocorre de duas maneiras: transmissão horizontal, em que o animal ingere alimentos contaminados com oocistos; e a transmissão mamaria e transplacentária (ou vertical), ocorre quando o filhote se infecta com taquizoítos transmitidos diretamente da matriz reprodutora [28]. A transmissão vertical é considerada a principal forma de manutenção do parasito nos rebanhos e está associada a diversas perdas econômicas, uma vez está entre as principais causas de aborto [28, 38]. Conforme demonstrado na Figura 1, o ciclo infeccioso deste parasito é composto por três estágios: taquizoítos, cistos teciduais e oocistos. Os oocistos são eliminados no meio ambiente através das fezes dos animais infectados; já os taquizoítos, estágio de rápido crescimento e acelerada disseminação, são formados no hospedeiro intermediário após ingestão dos oocistos; e, por fim, os cistos teciduais são formados após a replicação endógena dos taquizoítos, infectando células além da hospedeira e despertando resposta imune inata e adaptativa [39].

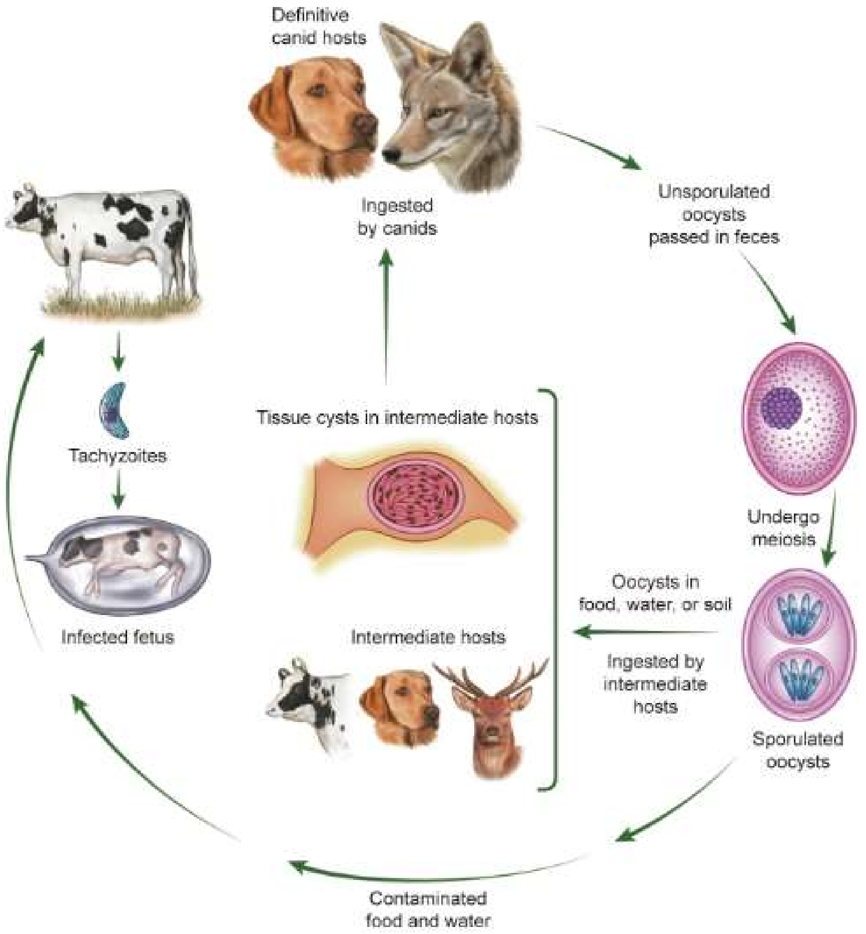

Figura 1 - Representação esquemática do ciclo de transmissão da neosporose bovina através dos hospedeiros intermediários e definitivos. A figura foi publicada em artigo de autoria de Khan e colaboradores [39].

Atualmente, a análise epidemiológica dentro de um exame não invasivo ajustado a múltiplas amostras é um desafio, exigindo mecanismos inovadores capazes de fornecer informações sobre as rotas de infecções e riscos de rebanho [28, 40]. Os fazendeiros usam a anamnese, a avaliação histórica dos animais e a percepção dos sinais clínicos como diagnóstico *in loco* [38]. No entanto, nas infecções crônicas, os sinais clínicos são quase indetectáveis, dificultando a divulgação da doença e aumentando sua disseminação [37].

A identificação sorológica frequentemente busca alvos antigênicos da superfície celular de *Neospora caninum* (NcSAG) ou proteínas cujas sequencias estejam relacionadas às de superfície (NcSRS) [41]. A proteína 10 do micronema (NcMIC10) e a proteína granular 7 (NcGRA7) também são alvos de estudo para atuação como biomarcadores na detecção da doença [40, 42]. Todavia, estes procedimentos testam os antígenos contra soros positivos, negativos e infectados por toxoplasmose para estabelecer um diagnóstico seguro. Logo, estes estudos desconsideram uma série de patógenos geneticamente similares ou cuja sintomatologia ao infectar os ruminantes se assemelhem as apresentadas pelo quadro de neosporose.

Em muitos casos, os animais infectados pelo *Neospora caninum* são erroneamente diagnosticados e tratados contra toxoplasmose [40]. Porém, um grupo de pesquisa do Reino Unido estudou a prevalência de bovinos infectados naturalmente com *Toxoplasma gondii* e revelou que apenas 1,79%, em um grupo de 305 animais, são portadores da doença [43]. Outro estudo indica a baixa presença nos tecidos do parasito, mesmo em animais infectados experimentalmente [44]. Portanto, a toxoplasmose bovina é um fenômeno raro em bovinos naturalmente infectados e geralmente a infecção não está associada ao aborto. Existem muitas pesquisas que indicam semelhanças entre as infecções induzidas por *N. caninum*, *Sarcocystis spp.* e *Besnoitia besnoiti* [45-47]. No entanto, até o momento, um único estudo avaliou a frequência de anticorpos anti-*Besnoitia besnoiti* em ruminantes em toda a América do Sul [48]. Segundo os autores, os baixos títulos detectados não correspondem aos já descritos na África e na Europa, sendo necessárias mais investigações para caracterizar a infecção em território brasileiro. Vale ressaltar também que relatos observados na literatura indicam que infecções por esse organismo levam a alterações bioquímicas no sangue [43], queda na produção de leite [44] e falhas reprodutivas [49], não havendo relatos evidentes que indiquem a relação destas infecções ao aborto.

Já o protozoário *Trypanosoma vivax* é considerado um dos principais causadores de falhas reprodutivas em bovinos devido à redução da libido, indução de um estado febril e aumento do volume ejaculado, acarretando no baixo desempenho sexual destes animais [25, 50]. Segundo o NCBI, o Trypanosoma vivax é um organismo celular pertencente ao reino *Eukarya*, clado *Discoba*, classe *Kinetoplastea*, ordem *Trypanosomatida* e família *Trypanosomatidae* (NCBI:txid5699). Estes protozoários atuam de maneira diferentes nos animais macho e fêmea, sendo que nos primeiros, as consequências da infecção levam a má qualidade do sêmen, redução da libido e retardamento da puberdade; e nas fêmeas, levam a subfertilidade, efeito patogênicos no feto e ciclos estrais anormais [50]. O primeiro relato descrito indicando a presença deste parasito no Brasil é de 1972, após um surto nos rebanhos de búfalos no estado do Pará [51]. Atualmente, anticorpos anti-*Trypanosoma vivax* já foram detectados em bovinos de mais de 14 estados brasileiros [52-54].

A infecção pode ocorrer por dois modos: a transmissão pela mosca africana tsé-tsé (transmissão cíclica), e a mecânica, por fômites contaminados ou insetos hematófagos, como dípteros sugadores de sangue [55]. A transmissão cíclica é mais restrita a países africanos ou locais em que há a presença do mosquito vetor, que multiplica os tripanossomatídeos epimastigotas em sua glândula salivar [56]. Já nos rebanhos da América do Sul, devido à ausência das moscas tsé-tsé, o principal mecanismo de propagação dos protozoários metacíclicos, já em estágios infecciosos, se dá através de moscas hematófagas que carregam o parasito até a corrente sanguínea do animal [55]. A tripanossomíase pode causar infecções agudas e crônicas em ruminantes, que podem resultar em graves alterações hematológicas, perda da condição corporal, diminuição da produtividade e alterações na reprodução [50, 57]. Diferentes sinais clínicos inespecíficos se manifestam no animal, dentre eles vale destacar a anemia, fraqueza progressiva, conjuntivite, febre, letargia, diminuição da produção de leite e carne, eventualmente morte [55, 57, 58].

Os testes sorológicos atuais são geralmente compostos por antígenos brutos obtidos de sangue de bovinos infectados experimentalmente, o que leva à baixa especificidade [59]. O antígeno de região repetida GM6 foi usado para diagnosticar a tripanossomíase em amostras de campo infectadas e proporcionou uma sensibilidade de 91,5% e especificidade de 91,3% em uma comparação com todo o lisado de tripanossoma ELISA [59]. Fleming [60] também utilizou uma abordagem sorológica para testar a antigenicidade de dois antígenos encontrados por meio de análise proteômica, atingindo 92-94,5% de sensibilidade e 88-89,8% de especificidade usando apenas amostras infectadas e não infectadas. Uzcanga [61] utilizou a proteína p64, anteriormente descrita em *T. congolense*, como um antígeno em teste do tipo ELISA e obteve 71,6% e 98,8% de sensibilidade e especificidade, respectivamente. No entanto, um dos desafios dos testes sorológicos é a existência de reações cruzadas entre antígenos imobilizados com o soro de bovinos infectados com outros patógenos, o que pode diminuir a especificidade da interação e limitar o diagnóstico [62].

Os tripanossomos desenvolvidos no processo infeccioso se mantêm na corrente sanguínea modulando a resposta imunológica, mas também afetando o funcionamento de órgãos vitais do animal [63]. No entanto, esta ação do protozoário é mais amena em bovinos que se mostram mais resistente a infecção, ou seja, alguns animais apresentam uma resposta imunológica mais eficiente no controle da parasitemia e dos principais sintomas. Este fenômeno de resposta e controle dos sinais clínicos é conhecido como tripanotolerância [64]. Embora não sejam susceptíveis a um quadro infeccioso agudo, estes animais são agentes importantes no controle e transmissão da doença, sendo responsáveis pela manutenção silenciosa do protozoário no rebanho. Os animais infectados além das alterações fisiológicas, sofrem modificações em parâmetros bioquímicos do sangue [56] e no quadro de citocinas, uma vez que nos primeiros dias após a infecção nota-se um aumento na produção de citocinas pró-inflamatórias e, em infecções crônicas, percebe-se o aumento das citocinas anti-inflamatórias [63]. Compreender e conhecer todos os aspectos envolvidos no processo infeccioso do animal é de grande valia para o desenvolvimento de técnicas de terapia e diagnóstico mais acuradas e eficientes.

JUSTIFICATIVA

As principais técnicas aplicadas no diagnóstico da tripanossomíase bovina se baseiam na percepção dos sinais clínicos ou na detecção do parasito em amostras de esfregaços sanguíneo [59]. Porém, a detecção de protozoários por métodos parasitológicos é uma tarefa difícil, uma vez que a ocorrência da doença em bovinos no Brasil é considerada crônica e assintomática. Por outro lado, os testes sorológicos fornecem informações úteis quanto ao contato com o patógeno, além de ser facilmente aplicado em um grande número de amostras e para avaliar com mais sensibilidade a presença do parasito e os efeitos da infecção na saúde animal, produtividade e risco de doença. No entanto, nos desafios para o desenvolvimento de um bom teste sorológico estão incluídas as características e a qualidade dos antígenos utilizados.

Assim como para tripanossomíase, o diagnóstico da neosporose bovina é feito a partir da percepção dos sinais clínicos, avaliação do histórico do animal e anamnese [32, 38]. Entretanto, ao longo dos anos, vários estudos buscaram aprimorar a detecção sorológica e molecular do protozoário e/ou seus componentes. Tanto a Reação em Cadeia da Polimerase (PCR, do inglês *Polymerase Chain Reaction*) convencional quanto em tempo real são técnicas refinadas que vêm sendo utilizadas na detecção do material genético do parasito em diferentes tecidos, como cérebro, coração e placenta [65].

Até o momento, não existem medidas terapêuticas unânimes para o tratamento da tripanossomíase bovina e o principal medicamento para controle do protozoário vem sendo utilizado há mais de 60 anos sem impedir a propagação e a erradicação da doença [66]. Já para a neosporose, pesquisadores buscaram utilizar como medida terapêutica o Triclosan, agente inibidor da enoyl acyl redutase, que atua limitando o crescimento do protozoário [67]; e o anticorpo monoclonal baseado na proteína 14-3-3 como estimulante de citocinas pelo sistema imune [26]. No entanto, nenhum tratamento eficaz ou vacina estão disponíveis no mercado [68]. Por este motivo, o controle dos animais infectados é realizado principalmente pelo manejo, isolamento e abate para que se evite a propagação ao longo do rebanho. Neste sentido, o estabelecimento de plataformas seguras e de baixo custo para o diagnóstico destas protozooses representa um avanço na produtividade do animal, além de amenizar os danos econômicos desencadeados pela infecção.

Recentemente, estudos vêm se dedicando a estabelecer métodos eficientes de diagnóstico aplicáveis diretamente nos locais de infecção, os chamados testes point-of-care (POC). Estes testes buscam consolidar ferramentas que sejam rápidas e de fácil aplicação, além de apresentarem baixo custo operacional [69]. O desenvolvimento de ferramentas POC será essencial no combate da disseminação e no controle de doenças infecciosas em uma população na qual condições laboratoriais são precárias [70]. Por isso, os ensaios imunológicos de fluxo lateral se desenvolveram atendendo os requisitos de um teste POC e estão imobilizando proteínas, nucleotídeos e/ou seus fragmentos para ensaios *in loco* [69]. Neste sentido, o avanço no desenvolvimento de moléculas naturais ou sintéticas que atuem na detecção, com elevada especificidade e acurácia, destes patógenos bovinos irá alavancar o desenvolvimento dos testes POC aplicados na prática clínica, facilitando a análise do status dos rebanhos de pequenos e grandes produtores.

DELINEAMENTO DO TEXTO

Para estruturar a presente tese, decidiu-se por dividi-la em três capítulos e quatro anexos que serão detalhados brevemente abaixo a fim de guiar a leitura deste material para torná-la mais dinâmica:

- Capítulo 1: a execução deste primeiro capítulo iniciou-se em 2017, ao longo do meu mestrado. Neste período, utilizamos a triagem por phage-display para selecionar peptídeos e proteínas que identificassem com precisão a tripanossomíase bovina em análises sorológicas. Ao fim desta etapa, havíamos identificado vários peptídeos e uma proteína potencial. No doutorado, optamos por seguir com a proteína como antígeno e só foi possível concluir os experimentos sorológicos e expandir o volume de soro para validação estatística ao longo do primeiro ano de doutorado. Como resultado desta seção, obteve-se um artigo científico, submetido para análise na revista

Applied Microbiology and Biotechnology (Fator de impacto atual de 3,53), e um depósito de patente (número do depósito BR 10 2019 003717 2).

- Capítulo 2: neste segundo capítulo, seguimos com a motivação em buscar novas moléculas para diagnóstico sorológico para protozooses bovinas. No entanto, o modelo de doença utilizada nesta seção foi a neosporose bovina. Aqui, buscamos utilizar uma estratégia de triagem totalmente computacional para diminuir os custos envolvidos na seleção de epítopos por phage-display. Como resultado desta seção, construímos uma proteína sintética patenteada (número do depósito BR 10 2019 003721 0) e publicamos um artigo científico na revista Journal of Clinical Microbiology (Fator de impacto de 5,897).
- Capítulo 3: nesta seção, buscamos estabelecer um protocolo de baixo custo para seleção de aptâmeros de DNA contra alvos proteicos imobilizados. Para tanto, decidimos utilizar a proteína sintética desenvolvida no Capítulo 2, para a posterior avaliação destes aptâmeros no diagnóstico da neosporose bovina. Se tratando de uma tecnologia emergente e ainda pouco explorada em nosso país, acreditamos que o estabelecimento do protocolo de seleção de aptâmeros motivará a busca por metodologias alternativas para o diagnóstico molecular de diferentes doenças relevantes em nosso cenário nacional e regional. Em um futuro próximo, visualizamos o depósito de patente para proteção da(s) molécula(s) selecionadas(s) e publicação de um artigo científico.
- Anexo 1: neste anexo, inserimos um artigo científico de revisão, publicado na revista Frontiers in Microbiology (Fator de impacto de 4,076), no qual discutimos sobre a utilização de ferramentas moleculares emergentes, como aptâmeros e CRISPR, no diagnóstico de doenças infecciosas.
- Anexo 2: já neste anexo, disponibilizamos para análise um artigo científico escrito para a ser submetido na revista Process Biochemistry (Fator de impacto de 2,952), no qual abordamos e discutimos a aplicação de técnicas de biologia sintética na ciência dos alimentos, com foco na produção de alimentos e melhoria de aspectos nutricionais.

OBJETIVOS

Estabelecer metodologias alternativas e de baixo custo para selecionar e construir moléculas que atuem no diagnóstico sorológico e molecular de protozooses bovina. Assim sendo, os objetivos específicos são:

- A partir do mapeamento de antígenos por *phage-display* e da triagem computacional de epítopos, selecionar e expressar proteínas de *Trypanosoma vivax* que forneçam um diagnóstico acurado para a tripanossomíase bovina.

- A partir de dados genômicos, construir uma proteína sintética baseada na predição de epítopos lineares de células B que diferencie soros de animais infectados com neosporose bovina de animais saudáveis.

- Estabelecer protocolo para seleção de aptâmeros de DNA e caracterizar sondas que interajam com a proteína sintética construída para o diagnóstico da neosporose bovina.

CAPÍTULO I: ARTIGO SUBMETIDO NA REVISTA APPLIED MICROBIOLOGY AND BIOTECHNOLOGY

Title

Phage-display and immunoinformatic screening of antigenic protein applied on bovine *Trypanosoma vivax* serodiagnosis

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Abstract

Diseases such as bovine trypanosomiasis caused by the protozoan *Trypanosoma vivax* affect the herds, causing enormous damages to the cattle raising. In Brazil, bovine trypanosomiasis is a lifelong disease which available methods of diagnosis are insufficient to control the its spread. Bovine trypanosomiasis can be detected by parasitological, molecular or immunological diagnostic methods. Serological diagnosis holds valuable importance in disease control due to its adjustability in large scale-up tests in a short time at low-cost maintaining sensitivity and specificity close to molecular methods. In the present work, a phage display screening combined with a genome-scale immunoinformatic strategy was applied to predict and to select an antigenic protein with high potential for the serodiagnosis of bovine trypanosomiasis. The recombinant protein was used as antigen for an ELISA assay and statistical analysis showed 88.5% (± 9.1) sensitivity to identify infected animals. Moreover, antigen cross-reaction with other bovine diseases such as tuberculosis, neosporosis, leptospirosis, and brucellosis, showed 89.3% (± 7.9) specificity of. According to the kappa index, the diagnostic molecule had a substantial agreement with the molecular and parasitological approaches. We conclude that phage display combined with immunoinformatic screening strategy are a high-throughput method to select antigenic proteins that enhances serological diagnosis assays.

Keywords: *Trypanosoma vivax*, phage display, computational proteomics, immunoinformatic, serodiagnosis.

Key Points:

Introduction

Trypanosomiasis is a disease that affects animals including cattle, sheep, buffaloes and other biungulates, occurring mainly in the African continent (Baral 2010).

The disease is caused by hemoflagellate protozoans such as *Trypanosoma brucei*, *T. congolense*, and *T. vivax,* the latter one of the most aggressive species commonly associated with symptoms like fever, anemia and weight loss (Fleming et al. 2016; Osorio et al. 2008). *T. vivax* infects wild and domestic animals, especially in African and South American countries (Batista et al. 2012; Osorio et al. 2008).

The routes of infection of *T. vivax* are different in Africa and America (Osorio et al. 2008). Through mechanical transmission, a major problem in Brazil, the disease settled in several regions of Central and South America despite the presence of the biological vector (Fleming et al. 2016). In this case, the mechanical transmission of pathogen is associated to tabanids or use of contaminated needles for oxytocine application right before milking. *Trypanosoma vivax* protozoan negatively impacts Brazilian livestock, decreasing animal productiveness (Batista et al. 2012). Infected animals manifest acute and chronic disease, which may be associated with hematological disturbances, weaknesses, sterility, abortions, respiratory illnesses, and reproductive failures (Adamu et al. 2007; Osorio et al. 2008; Uzcanga et al. 2016).

Some diseases share same symptoms in cattle raising. Bovine trypanosomiasis symptomatology is nonspecific and, in many cases, become a silent disease, as the symptoms are also common for other diseases, such as tuberculosis, neosporosis, leptospirosis, and brucellosis (Boulange et al. 2017). So far, diagnosis is still based on clinical signs or blood smears (Pillay et al. 2013). Molecular and immunological techniques are alternative to parasitological methods. However, the molecular approaches demand high cost and specific training for handling samples and equipment (Bossard et al. 2010). Serology is a useful tool applied in large scale epidemiological studies to detect anti-*Trypanosoma* antibodies that facilitates disease control campaigns (Bossard et al. 2010). The development of rapid, low-cost and efficient serological methods for trypanosomiasis depends on the availability of parasite targets to enable the identification of the infection by high-affinity binding between host antibodies and parasite proteins with high specificity and sensibility. In many cases, the search for this target is the limiting factor (McGuire et al. 2009).

Phage display is a cloning technique capable of isolating and selecting peptides that mimic linear and conformational epitopes (mimotopes) that can be used to discover new antigens (Fack et al. 1997). The identification of reactive peptides on the phage surface allowtheir use and/or the subsequent synthetic construction with directed application for diagnosis and/or development of vaccines against various diseases (He et al. 2012; Toledo-Machado et al. 2015a; Toledo-Machado et al. 2015b). In addition, immunoinformatic screening approaches enable an exhaustive search of proteins encoded in the parasite's genome and contribute to the rapid identification of potential antigens In a more effective way, spending less time and costing less (Colinge and Bennett 2007).

The aim of this work was to identify an antigenic protein in the *T. vivax* genome for the serological diagnosis of bovine trypanosomiasis. Initially, a subtractive selection of phage display identified peptides that reacted to trypanosomiasis-infected sera. Then, an immunoinformatic screen identified a potential antigen which was used to improve the serodiagnosis.

Materials and methods

Bovine sera samples and ethics statement

Sera from healthy bovine (n=31) and infected animals (trypanosomiasis-infected group, n=26, tuberculosis-infected group, n=6, neosporosis-infected group, n=6, leptospirosis-infected group, n=7, and brucellosis-infected group, n=6) were collected in farmed animals in Uberaba (19°44′53″S and 47°55′54″W), Viçosa (20°45'17"S and 42° 52'57"W), Montes Claros (16°44′13″S and 43°51′53″W), and Curvelo (18°44'57"S and 44°26'48"W), Southern Brazil. The sera were previously characterized by molecular techniques with primers previously reported to confirm mono-infection with (Cadioli et al. 2015; Chisi et al. 2017; Hamond et al. 2014; Lally et al. 1996; Sweetline Anne et al. 2017). Ethics approval was given by The Animal Experimentation Ethical Committee from the Universidade de Uberaba, (CEEA/UNIUBE, protocol number 001/2013). Samples collection followed ethical principles of cattle experimentation procedures.

Trypanosoma vivax infection was confirmed by both parasitological and serological test through microscopic identification of pathogens in blood smears and detection of anti-*T. vivax* IgG by indirect immunofluorescence assay (IFA), respectively. *Leptospira sp.* infection was confirmed by microscopic agglutination test (MAT). *Neospora caninum* infection was identified by IFA and confirmed by tissue immunohistochemistry. Brucellosis and tuberculosis infected animals were tested respectively by the Serum Agglutination Test (SAT) using intradermal tuberculin, and both pathogens confirmed by PCR. Healthy animals' sera were confirmed by negative results in parasitological, serological and PCR tests.

Phage display selection and titration of clones

Figure 1 represents the protocol for the three steps of biopanning cycles (incubation, elution, and amplification) until the phage selection following analyses. The phage library containing random peptides fused to the minor coat protein of M13 filamentous phage (Ph.D.-C7C library; New England BioLabs, USA) were used in this study, as described previously (Costa et al. 2014). Briefly, bio-panning was performed using IgGs that had been purified from healthy bovines. The purification of bovine IgG antibodies from the serum samples of the different groups was performed by coupling to magnetic beads conjugated to protein G (Dynabeads, Invitrogen). The supernatant containing the non-adherent clones was recovered and transferred to a new tube, titrated, and used in the positive selection. In this step, the previously recovered phage clones were transferred to a tube containing IgGs purified from serum samples of *T. vivax*-infected bovines and incubated for 30 min at room temperature. The supernatant was then removed and the remaining phages bound to the IgGs were washed 10 times with 1 mL of TBS-T and eluted with 500 µL of 0.2 M glycine buffer (pH 2.0). Next, 75 µL of 1 M Tris-base (pH 9.0) was added to neutralize the acidic pH. The recovered phage clones were used in a new biopanning cycle and the process was repeated three times. Finally, after the 3rd cycle, the selected phage clones were recovered and titrated. Phage clones were diluted 10^{-1} to 10^{-11} in 500 µL of sterile phosphate buffered saline (PBS), mixed with a culture of *Escherichia coli* with $OD_{600\ nm}$= and plated on LB agar plates containing 1 mL of IPTG 1.0 mM (isopropyl-β-D thiogalactopyranoside). The colonies were individually quantified, and the titration was performed for each biopanning cycle. A total of 96 colonies were selected from the plate and added to 200 µL of LB in a sterile culture plate, and then the plate was sealed and incubated for 5h with constant stirring at 37°C. After incubation, the plate was centrifuged for 20 min at 2,250 x g and the supernatant was transferred to a new plate, in which a polyethylene glycol (PEG)- NaCl (20% PEG 8000 and 2.5 M NaCl) solution was added (1/6 of the total volume of the supernatant) following incubation for 16 h at 4°C. Finally, the plate was centrifuged for 1h, the supernatant was removed, and the pellet was resuspended in 500 µL of a solution composed of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 4 M NaI. DNA of each selected phage clone was extracted and sequencing by Sanger method as described previously (Costa et al. 2014).

Phage-ELISA

Previous titration curves were performed to determine the most appropriate phage concentration and the antibody dilution to be used. For the Phage-ELISA, clones diluted in 100 µL of coating buffer (50 mM carbonate buffer, pH 9.6) were coated (1×10^9 phages per well) onto 96-well microplates (Nalgen Nunc International, USA) for 16 h at 4º C. A wild-type phage (1×10^9 phages per well) was used as a control. After the coating step, the plates were blocked with 150 µL of PBS containing 5% bovine serum albumin (BSA) for 1h at 37°C and then treated with 1:100 dilutions of bovine serum samples (non-infected or *T. vivax*-infected) for 1h at 37°C. Peroxidase-conjugated antibodies specific for bovine IgG (Sigma-Aldrich, USA) were diluted at 1:5,000 and added to the plates for 1h at 37°C. The wells were washed, and the 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich) in citrate buffer containing hydrogen peroxide was added to the plate. The plates were incubated for 30 min in the dark at 37°C. The reaction was halted by adding 4M H_2SO_4, and the $OD_{450\ nm}$ was read with an automatic microplate reader (EMax®; Molecular Devices, USA). Each serum sample was assayed in duplicate.

Immunoinformatic protein screening

To identify phage display peptides alongside antigenic proteins sequence, a search was carried out inputting the 25 peptides phage sequence as query to BLASTp tool (Altschul et al. 1990) against the predicted *T. vivax* proteome deposited in the GenBank database (Benson et al. 2005). The bioinformatics analyses were only based on proteins containing peptides with 100% of coverage and identity value above 70%. The BLASTp parameters were adjusted for the input of short sequences: expected limit of

100, maximum match in query interval 0, BLOSUM62 matrix, costs for extension gap 11 and existence 1, and used a conditional scoring matrix adjustment. The BepiPred 1.0 server (Larsen et al. 2006) was used to identify potential antigenic proteins using a medium score higher than 1.3, which ensure specificity of 96% in the prediction of a true epitope. The level of expression of each protein-encoding gene in trypomastigotes were recovery from Jackson (Jackson et al. 2015) and they were represented as Reads Per Kilobase per Million (RPKM). The potential specificity of identified epitopes was evaluated using Blastp (Altschul et al. 1990) implemented in the NCBI database and with default parameters against other bovine pathogens such as *T. brucei* (Jackson et al. 2010), bovine leukemia virus (BLV) (Dao et al. 2018), *Leptospira spp*. (Xu et al. 2016), *Neospora caninum* (Reid et al. 2012), *Babesia bovis* (Brayton et al. 2007), *Theileria annulata* (Pain et al. 2005), *Brucella abortus* (Garofolo et al. 2015), and *Mycobacterium bovis* (Guimaraes et al. 2015).

Gene amplification and cloning

A total of 100 ng of *T. vivax* DNA was used as a template to amplify the selected protein coding region with 0.5 mM of each primer (for CCD19908.1: Forward: 5’-atgagtcggctgcacggc-3’; Reverse: 5’-ccacacaggtcacagcaacacact-3’; for CCC52526.1: Forward: 5’-atgcggcgcgttttgctttcg-3’; Reverse: 5’-cgcaacgaacaacaggcgct-3’), and 1 U of GoTaq® DNA polymerase (Promega Biotecnologia do Brasil Ltda). The PCR products were evaluated by electrophoresis on 1% agarose gel and DNA fragments were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega Biotecnologia do Brasil Ltda) and quantified by the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, EUA). Then, amplicons were inserted into the cloning vector system pGEM-T Easy (Promega Biotecnologia do Brasil, Ltda) and transformed into *Escherichia coli* XL1-

Blue competent cells through electroporation in a 1 mm cuvette under 1.8 kV, 25 µF, and 200 Ω. The *Nhe*I e *Xho*I enzymes were used to cleave and transfer the cloned fragment to the pET28a expression system with T4 DNA ligase. The ligation product was transformed into *Escherichia coli* Arctic Express through electroporation as described above. PCR with T7 initiator and terminator primers and Sanger DNA sequencing were used to confirm the cloning..

Expression and purification of recombinant protein

The bacterial expression system was induced with 0.4 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 24 h at 12 ºC. Next, the culture was pelletized and resuspended in PBS. The bacterial extract was mixed in a buffer containing 2-mercaptoethanol, heated at 95°C for 10 min, and submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to verify protein pattern expression. Resolving gel was prepared in 14% concentration with 1.5 M Tris-HCl, pH 8.8, 30% acrylamide, 10% SDS, and 10% ammonium persulfate and TEMED. Samples were mixed with loading buffer (10% SDS, 0.5 mM Tris-HCl 0,5 pH 6.8, 1% bromophenol blue, 5% 2-β-mercaptoethanol, and 50% glycerol), and stained with Coomassie Brilliant Blue R-250. For Western-blotting (WB) analysis, the proteins were transferred to a nitrocellulose membrane and blocked with 5% w/v BSA, at room temperature for 1 h. The nitrocellulose membrane was washed three times with PBS buffer supplemented with 0.5% Tween 20 (PBS-T) and mouse anti-His antibody 1:5,000 v/v (GE HealthCare, USA) was added and incubated by shaking for 2 h at room temperature. Then, the membrane was washed repeatedly and incubated with secondary antibody peroxidase-conjugated (1:15,000 v/v, GE HealthCare, USA) for 3 h at room temperature. The revelation occured through a reaction between his-tagged protein-antibody complex and 3,3’-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, USA) substrate, catalyzed with H_2O_2 for 10 min. That reaction was protected from light.

Bacterial culture was sedimented, resuspended in PBS buffer supplemented with 30 mM imidazole and 8M urea, and incubated in ice for 30 min. The bacterial cellwall was disrupted by sonication to evaluate soluble and insoluble components in purification columns. The recombinant protein was purified in affinity chromatography HisTrap HP histidine-tagged protein purification columns (GE HealthCare, USA). The column was washed five times with buffer A (20 mM Na_2HPO_4, 500 mM NaCl and 30 mM imidazole) and proteins were eluted with a gradient that ranges from 0 to 100% of buffer B (20 mM Na_2HPO_4, 500 mM NaCl and 500 mM imidazole). Purified protein fractions were analyzed by SDS-PAGE and the residual urea was removed with a 20 kDa pore-sized dialysis membrane..

Protein-ELISA and statistical analysis

ELISA was performed in Nunc MaxiSorp flat-bottom multi-well plates (ThermoFisher Scientific, EUA). The plates were coated with 2 μg of recombinant protein per well, diluted in 1 M carbonate buffer (pH 7.3) and incubated overnight at 4ºC. Next, plates were washed five times with a washing buffer containing PBS (pH 7.2) and 0.5% Tween 20 (Sigma-Aldrich, USA). Blocking buffer (PBS with 5% v/v BSA protein) was added and the wells incubated at 37ºC for 1 h. An additional wash step was carried out to remove residual blocking buffer. Bovine sera were diluted 1:300 w/v in washing buffer and plates were incubated at 37ºC for 1 h. Then, anti-bovine IgG antibody 1:5000 v/v, coupled with horseradish peroxidase, was added and incubated for 1 h at 37ºC. O-phenylenediamine dihydrochloride substrate (OPD, Sigma-Aldrich, USA) was diluted in a buffer containing 0.1 M citric acid, 0.2 M Na_2PO_4, and 0.01%

H_2O_2 (pH 5.0). Plates were kept in the dark, for 30 min, at room temperature. Finally, The OD was measured at 492 nm with an ELISA reader (SpectraMax M -Molecular Devices, Washington, USA).

Statistical analyses were performed using GraphPad Prism™ version 5.0. The lower limit of positivity (cut-off) for the infected sera was established for optimal sensitivity and specificity using the Receiver Operator Curve (ROC curve). Test performance was evaluated according to the sensitivity, specificity, and area under curve (AUC). A nonparametric One-Way ANOVA test was used to compare means of absorbance between non-infected and infected groups. The differences were considered statistically significant at $p < 0.001$. Additional analytical parameters, such as Positive and Negative Predicted Value, Accuracy and Kappa-index were calculated to provide powerful mathematics support.

Results

Screening phage clones and selection of antigenic proteins

Positive selection of phage clones was realized by the acceptance of bovine sera antibody samples with *T. vivax*. Reactivity of selected phage clones against trypanosomiasis-infected sera was evaluated by a phage-ELISA (Supplementary Figure S1). Coming out of previous step, 25 clones were selected and they had their sequence amplified and identified, as shown in Table 1.

Thereupon, after selecting phages with highest reactivity potential, genome of *T. vivax* was mapped in search of antigenic proteins. Peptides were ranked according to i) query cover equal to 100%; ii) higher identity value; iii) medium predictive epitope score of 1.3; iv) reported gene expression level, according to RPKM. The peptide QSTSGSS met all eligibility criteria since it reached, concurrently, the highest query cover and identity value. Therefore, the proteins CCD19908.1 and CCC52526.1 that had the aminoacids QSTSGSS were expressed in a heterologous system and used in a serological diagnosis.

Recombinant protein expression, purification and epitope analysis

A 700 bp DNA fragment corresponding to the protein CCC52526.1 was amplified from the *T. vivax* genome (Figure 2A). An amplicon of 500 bp indicating an undesired amplification was also seen but did not compromise gene-target purification. On the other hand, a DNA fragment around 3000 bp was expected upon amplification of the gene encoding the protein CCD19908.1, which was not seen (Figure 2A). Consequently, further studies were done with the protein CCC52526.1.

Gene cloning was confirmed by Sanger DNA sequencing and protein was expressed in *E. coli* Artic Express(Figure 2B). The concentration of all samples was equivalent but a slight difference was seen on the induced sample near the 25 kDa protein marker,, indicating the expression of the protein of interest. In order to validate the expression of the protein CCC52526.1 a western blotting using anti-his antibody was carried out and a specificband indicating the production of the desired protein was seen (Figure 2C). Affinity chromatography protein purification was successfully performed in a measurement of purified fractions against flow-through (FT) samples (Figure 2D). The aminoacid sequence of the protein CCC52526.1 was compared to other pathogens (Table 2). Some epitopes sites, such as ETDTTGS and SVTPGAE, had large similarity with almost all pathogens but bovine leukemia virus.The complete protein sequence presented 53% e 28% of identity with *T. brucei* and *B. bovis*, although sequence similarities did not cover epitopes predicted regions.

Validation as a diagnostic target

To evaluate the diagnostic potential of the new antigen, an indirect ELISA using the recombinant protein CCC52526.1 as antigen for detection of infected animals' sera (Figure 3). A total of 3/26 of the trypanosomiasis infected samples were below the cut-off line, providing a sensibility of 88.5% for the diagnosis of *T. vivax*. On the other hand, 6/56 tested sera, including healthy and non-trypanosomiasis infected sera, were over the cut-off edge, resulting in specificity equal to 89.3%. Receiver Operating Characteristics (ROC) curve showed the relationship between sensibility and specificity for this antigen (Figure 3). Statistical analysis provided a Positive Predicted Value (PPV) and a Negative Predicted Value (NPV) of 74.2% and 94.1%, respectively, indicating a high probability that the disease is not present when the test is negative. Furthermore, the analysis suggested good accuracy and a substantial agreement among kappa-index value and a gold standard histopathological assay, equivalent to 86.6% and 0.705, respectively. Nonparametric One-Way ANOVA test with p-value lesser than 0.001 was calculated on the comparison with trypanosomiasis and further groups obtaining statistical support to the ability of antigen to discriminate samples from *T. vivax* infected bovines.

Discussion

From the expansion of sequencing tools, manipulation and study of genomes, access to DNA sequences of several pathogenic organisms have become common. Hence, the development of diagnostic tests for infectious diseases using resources that involve genomics, transcriptomics, metabolomics and, mainly, proteomics have been increasing (Manzoni et al. 2018). The purpose of the present work was to select an antigenic protein capable of identifying antibodies anti-*Trypanosoma vivax* in sera sample through a proteome screening based on phage display and immunoinformatic tools.

A serological analysis is a useful tool for large-scale epidemiological studies, facilitating disease control (Bossard et al. 2010). In addition to presenting well-established tests, there are direct applications in the diagnosis of infectious, autoimmune, and allergic diseases (Fierz 2004). These tests allow the rapid detection of infection, are non-invasive, can be quantitative, and are easily automated, enabling the evaluation of numerous samples simultaneously (Menezes-Souza et al. 2015). According to Bakari et al.(2017), biochemicals and hematological sera factors are critical tools in the immunological disease's diagnostic and prognostic. So, the development of new tests based on recombinant protein technology decreases the cost of technology and easily large-scale application (Bossard et al. 2010). General parasitological techniques provide high specificity but are arduous methodology and unsuitable to epidemiologic vigilance and periodic control (Toledo-Machado et al. 2015b). Crude antigen ELISA is a conventional and standardized technique for the diagnosis of trypanosomiasis, despite the need to manipulate the pathogen and smaller sensitivity than recombinant antigens (Mossaad et al. 2019). The use of recombinant antigens instead of whole antigens reduces the live parasite processing and allows better specificity control, eliminating shared antigens with others organism (Menezes-Souza et al. 2015). The choice of recombinant proteins as an antigen in diagnostic approach exemplifies an interesting strategy for field animal diseases, such as bovine trypanosomiasis. Thus, antigen selection is a critical step for serological methods because is directly related to specificity and sensitivity of the test. The immunoinformatics applied on epitope mapping decreasethe price of experimental analyses and potentialize the detection of true epitopes in therapeutic, diagnostic and vaccinal approaches (El-Manzalawy and Honavar 2010; Menezes-Souza et al. 2014).

Bovine trypanosomiasis can be diagnosed through histological techniques that recognize the parasite in blood smears, molecular techniques or immunological assays that detect the presence of antibodies produced during the infection (Bossard et al. 2010; Boulange et al. 2017). However, there is a lack of diagnostic methods applied to field samples, hindering the disease control (Pillay et al. 2013). The full absent or the difficulty in perceiving infected animals' clinical signs facilitates the distribution, hinder the detection and disease control (Almeida Kde et al. 2012). There are no gold standard assays to diagnosis bovine trypanosomiasis (Uzcanga et al. 2016). although someauthors suggest the use of microscopic parasite identification as reference (Pillay et al. 2013)

The GM6 repeated-region antigen was used to diagnosis trypanosomiasis in field infected samples and showed 91.5% sensibility and 91.3% specificity in a comparison with the whole trypanosome lysate ELISA (Pillay et al. 2013). Tests based on whole trypanosome lysates provide fewer reproducibility and antigen stability, besides raising ethical concerns aboutthe use in living animals (Bossard et al. 2010). Fleming et al.(2016) also used a serological approach to test the antigenicity of two antigens found through proteomic analysis, reaching 92-94.5% of sensitivity and 88-89.8% of specificity using only infected and non-infected samples. Uzcang et al. (2016) used the protein p64, formerly identified in *T. congolense*, as an ELISA antigen with 71.6% and 98.8% of sensitivity and specificity, respectively. Beyond that, when comparing the efficiency of diagnostic assays, the author suggests a substantial agreement between molecular techniques and p64-antigen based ELISA. Our statistical parameters indicate an equivalence in diagnostic efficiency when compared to recombinant proteins cited above. Moreover, according to Landis and Koch (Landis and Koch 1977), based on Kappa index equal to 0.705, there is a substantial agreement among the response of our ELISA assay and molecular/histological reference tests.

Along with the computational screening, we performed an alignment of linear epitopes sequence with closely related pathogens (Table 2). In individual analysis, the sequence identity is inversely associated with epitope size, once the identity values were tinier to extensive epitopes. On the other hand, the whole protein sequence did not present significant similarities to most of the pathogens analyzed, except with *T. brucei* and *B. bovis*. Nevertheless, immunological assay indicates that epitope combination along protein sequence overcome individual values in a diagnostic approach (Fig. 3). Some of the protein CCC52526.1 epitopes site were conserved in the *T. brucei* proteome. However, no concern was raised about differentiation at the species level of trypanosomes. To the best of our knowledge, this is the first work to use the most frequent pathogens in cattle farming to achieve an effective molecule to diagnosis trypanosomiasis in cattle.

Indirect ELISA measures antibody concentrations in a simple assay through colorimetric, fluorescent, and chemiluminescent reports, providing high throughput and reproducible analysis (Hnasko et al. 2011). The main disadvantage of immunoassay is a large number of false-positive results and unspecific protein immobilization on wells or membranes (Aydin 2015). Furthermore, cross-reaction between immobilized antigens and other bovine pathogens decreases interaction specificity edging the ELISA diagnosis (Almeida Kde et al. 2012). Bovine trypanosomiasis classical symptomatology remains unclear and unspecific, leading to an improper treatment to disease eradication (Cadioli et al. 2015). The infection virulence is associated with host trypano-tolerance, genetic and environmental factors, besides the animal nutritional aspects (Bittar et al.

2015) driving the animal to a mild or acute infection pattern (Baral 2010). The virulent amastigote protozoan can be observed only in African countries, wherever disease biological vector, the tsetse fly, are related (Bassi et al. 2018). In mild infections caused by trypomastigote protozoan, the better-described symptoms are weight loss, abortion or breeding failure, fever, weakness, hematological alterations, neurological and respiratory maladies (Batista et al. 2007; Cadioli et al. 2015; Masake et al. 2002). Those symptoms are closely related to the signs described from bovine field-infections such as bovine leukemia virus (Rodriguez et al. 2011), theileriosis (George et al. 2015), neosporosis (Almeria et al. 2017), leptospirosis (Cosate et al. 2017), tuberculosis (Domingo et al. 2014), brucellosis (Khan and Zahoor 2018), and babesiosis (Bal et al. 2016). Despite the meaningful diversity among *Trypanosoma vivax* surface protein expression profile from members of African *Trypanosoma* genus (Jackson et al. 2015), clinical signs presented in *Trypanosoma brucei* and *Trypanosoma vivax* infected-cattle are common (Blum et al. 2012).

In conclusion, we performed a massive screening of *T. vivax* predicted proteome using phage display and immunoinformatic tools to select an antigenic protein with high potential for immunodiagnosis of the bovine disease. The selected protein showed antigenically potential in the interaction with bovine infected sera, providing a discriminatory test against non-infected animals and/or infected with others abortive pathogens. So, the data indicates that the new antigen CCC52526.1 is specific and accurate for diagnosis of bovine trypanosomiasis. Moreover, finding a molecule to diagnosis a multitude of infectious disease could be easily performed based on workflows applied here.

Acknowledgment

The authors acknowledge the Diretoria de Tecnologia de Informação (DTI) from Universidade Federal de Viçosa to provide access to the computer cluster Jupiter for informatic analysis. The authors also acknowledge the financial support of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) provided as student and researcher fellowships during the project execution.

Compliance with Ethical Standards

Funding: This study was funded by FAPEMIG, CNPq and CAPES.

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: This article contains studies with animals approved by The Animal Experimentation Ethical Committee from The Universidade de Uberaba, (CEEA/UNIUBE, protocol number 001/2013).

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Figure legends:

Figure 1 Representative biopanning workflow steps for selection of reactive phage-clones with high target affinity against IgG anti-*Trypanosoma vivax* sera.

Figure 2 Expression and purification of the recombinant protein CCC52526.1 (A) Gel electrophoresis of PCR product amplified from the genomic DNA of *Trypanosoma vivax*. (B) Protein expression patterns in the wild-type (ctrl), non-induced, and induced systems. (C) Western-blotting analysis to confirm the expression of his-tagged protein CCC52526.1 : wild-type *Escherichia coli* Arctic Express was used as a control (ctrl), a transformed and non-induced system and induced one. (D) Affinity chromatography purification SDS-PAGE gel of the non-binding flow-through fraction (FT) and eluted purified fractions.

Figure 3 The diagnostic potential of the antigen CCC52526.1 for detection of bovines infected with *Trypanosoma vivax*. ELISA reactivity of sera collected from healthy animals(n=31), *Trypanosoma vivax*-infected (n=26), *Mycobacterium bovis* (n=6), *Neospora caninum* (n=6), *Leptospira sp*. (n=7), and *Brucella abortus* (n=6). *T. vivax*-infected samples were statistically different from other with p-value < 0.001 in One-Way ANOVA analysis. ROC curve conferring a relationship among sensitivity and specificity of the assay. a indicates p-value < 0.001.

Table 1 Mapping of phage-clone peptides sequences in predicted proteins from the *Trypanosoma vivax* proteome.

Phage peptide sequence	NCBI protein ID	Query Cover	Identity	*T. vivax* peptide sequence*	Initial coordinate	Final coordinate	Epitope prediction (score)	RNA level (RPKM) in trypomastigotes
PRSTSHL	CCC48034.1	100%	86%	PQSTSHL	53	59	PQST--- (1.552)	20.49
	CCC46635.1	100%	86%	PRSQSHL	776	782	PR----- (1.3615)	37.38
	CCC49077.1	100%	86%	PRSRSHL	785	791	PRS---- (1.5373)	18.08
LYHDTHY	CCC51335.1	100%	71%	LYEDTQY	136	142	-	39.34
NPFCGSR	CCC47882.1	100%	71%	DPFCGRR	117	123	-	14.59
FGAPVSL	CCC52531.1	100%	88%	FGAPLVSL	113	120	-	17.78
	CCC49152.1	100%	86%	FGQPVSL	315	321	-	17.96
	CCC49149.1	100%	86%	FGQPVSL	314	320	-	20.28
TSPPLHA	CCC51833.1	100%	86%	TPPPLHA	102	108	TPPPL-- (1.8474)	35.6
	CCC49444.1	100%	86%	TSPPRHA	1688	1694	TSPPRHA (2.006)	21.26
QSTSGSS	CCD19908.1	100%	100%	QSTSGSS	213	219	QSTSG-- (1.8774)	5.19
	CCC52526.1	100%	86%	QETSGSS	373	379	QETSGSS (1.7307)	14.73
TFSPLSV	CCD21738.1	100%	86%	TFSPQSV	570	576	-	10.76

	CCC46909.1	100%	86%	TFIPLSV	180	186	-	20.79
FPHLSRY	CCC53665.1	100%	71%	YPHLNRY	237	243	-	23.19
	CCC54210.1	100%	71%	FPHLAPY	554	560	-	28.05
	CCC49942.1	100%	71%	FAYLSRY	116	122	-	23.24
	CCC49646.1	100%	71%	FPDLSHY	113	119	-	20.54
NPFLADP	-	-	-	-	-	-	-	-
ALTPQLL	CCD21345.1	100%	71%	ALTAQLM	748	754	-	7.35
	CCC52718.1	100%	71%	AIAPQLL	163	169	-	15.94
MSPTYLL	CCC47119.1	100%	86%	MSPSYLL	106	112	-	17.57
	CCC49982.1	100%	71%	LSPSYLL	159	165	-	36.72
	CCC49677.1	100%	71%	MARTYLL	1000	1006	-	16.94
FPLFGLS	CCC48431.1	100%	86%	FPLFQLS	1202	1208	-	14.07
	CCD19296.1	100%	86%	FPLSGLS	5	11	-	18.16
	CCC53502.1	100%	71%	FSIFGLS	506	512	-	10.35
	CCD18084.1	100%	71%	FPMLGLS	477	483	-	42.98
	CCD19712.1	100%	71%	FSLFSLS	341	347	-	20.26
	CCC51989.1	100%	71%	FSLFSLS	194	200	-	12.78
DRAALSL	CCC49495.1	100%	86%	ERAALSL	22	28	-	41.33
	CCC49036.1	100%	86%	DRAALQL	579	585	-	24.53
	CCC48065.1	100%	86%	DMAALSL	1327	1333	-	17.68
	CCC48540.1	100%	71%	DRSSLSL	95	101	-	13.43
	CCD18528.1	100%	71%	ERAALSM	16	22	-	11.66
	CCC53799.1	100%	71%	ERAAISL	474	480	-	11.74
DLCTQIT	CCC50029.1	100%	71%	DLCASIT	400	406	-	17.15
GSRSYPR	CCC52735.1	100%	86%	GSRSQPR	480	486	GSRSQPR (1.4698)	17.03
	CCC46776.1	100%	71%	GSRPCPR	1333	1339	-	24.31
	CCC53956.1	100%	71%	GSVNYPR	379	385	-	18.67
	CCC50050.1	100%	71%	GSSVYPR	288	294	-	15.48
VQSWSVR	CCC46639.1	100%	86%	VQSLSVR	1554	1560	-	20.36
	CCD18696.1	100%	86%	VQSLSVR	137	143	-	4.04
	CCD20722.1	100%	71%	VRKWSVR	62	68	-	23.43
SPDSLFP	CCC47384.1	100%	71%	SPQKLFP	583	589	-	16.24
	CCC51526.1	100%	71%	SSNSLFP	856	862	-	14.71
	CCC50202.1	100%	71%	SSASLFP	39	45	-	22.13
PQDPKQW	-	-	-	-	-	-	-	-
PSPSRFP	CCC54313.1	100%	86%	PSPSRSP	785	791	PSRSP (1.5872)	14.68
	CCC48016.1	100%	71%	PSAARFP	15	21	-	33.9
	CCC52276.1	100%	71%	PAPSSFP	524	530	PAPSSFP (1.6685)	19.82
	CCC53034.1	100%	71%	PTPSPFP	214	220	PTPSPFP (1.6932)	24.52
DSAFRLK	CCC53794.1	100%	71%	DSGFRVK	115	121	-	19.0
	CCC48437.1	100%	71%	DPIFRLK	125	131	-	12.6
	CCC52697.1	100%	71%	DSVLRLK	184	190	-	37.96
AETVESC	CCC53168.1	100%	71%	AEAVEAC	484	490	-	31.22
	CCC50844.1	100%	71%	AETVQRC	646	652	-	38.39
	CCC48244.1	100%	71%	AETVNNC	1005	1011	-	11.79
VYSALVE	CCC51432.1	100%	86%	VYSQLVE	313	319	-	37.88
	CCC50426.1	100%	86%	VYSYLVE	336	342	-	17.31
	CCC47971.1	100%	71%	VYSELVD	535	541	-	23.31
	CCC47860.1	100%	71%	IYNALVE	322	328	-	25.31
GYSALVE	CCC50347.1	100%	71%	GYPTLVE	68	74	-	21.90
	CCC47351.1	100%	71%	GYAAFVE	984	990	-	22.05
	CCC49376.1	100%	71%	GYKAVVE	286	292	-	13.34
DRRGYGL	CCC53461.1	100%	71%	DRSTYGL	379	385	-	17.28
	CCC49297.1	100%	71%	DRRHFGL	458	464	-	9.47
ACFDGVF	CCC48332.1	100%	86%	AC-DGVF	216	221	-	32.26

* Red letters represent polymorphic amino acids compared to phage sequence.

Table 2 Predicted epitopes in the protein CCC52526.1 of *Trypanosoma vivax* and percentage of identity compared by BLASTp against proteins of other bovine pathogens.

Epitope	Score	*Trypanosoma brucei*	Leukemia virus	*Theileria annulata*	*Neospora caninum*	*Leptospira spp.*	*Mycobacterium bovis*	*Brucella abortus*	*Babesia bovis*
HDPTNDLTA	1.37	83.3%	66.7%	77.8%	66.7%	55.5%	53.8%	71.4%	77.8%
LDQPQRQPECDPGC	1.53	53.8%	28.6%	63.6%	42.8%	50%	56.2%	46.7%	45.4%
VDQETSGSSS	1.61	80%	40%	64.3%	60%	60%	85.7%	60%	71.4%
GDVTPCEGVSPCGGPLPHNEAERA	1.38	36.3%	33.3%	47.1%	37.5%	54.1%	52.2%	44.4%	50%
ETDTTGS	1.43	100%	NSS	100%	85.7%	85.7%	85.7%	71.4%	85.7%
GATGDGATIGAGEEECGVS	1.31	65%	31.6%	68.4%	47.4%	31.6%	52.6%	52.6%	36.8%
MTPLGEPYY	1.32	66.7%	55.6%	66.7%	77.8%	66.7%	55.6%	55.6%	66.7%
RGPSPVESGEGDNT	1.87	64.3%	42.8%	64.3%	64.3%	42.8%	64.3%	42.9%	64.3%
TENSDDP	1.81	85.7%	NSS	85.7%	85.7%	85.7%	71.4%	71.4%	71.4%
DSSQSEK	1.34	85.7%	NSS	85.7%	85.7%	85.7%	71.4%	57.1%	85.7%
SVTPGAE	1.25	85.7%	57.1%	85.7%	85.7%	85.7%	85.7%	71.4%	85.7%

Whole Protein	-	53%	NSS	NSS	NSS	NSS	NSS	NSS	28%

Supplementary figure:

Supplementary figure S1 - Validation of the potential of peptides identified by phage display for the diagnosis of bovine trypanosomiasis.

Figure 1

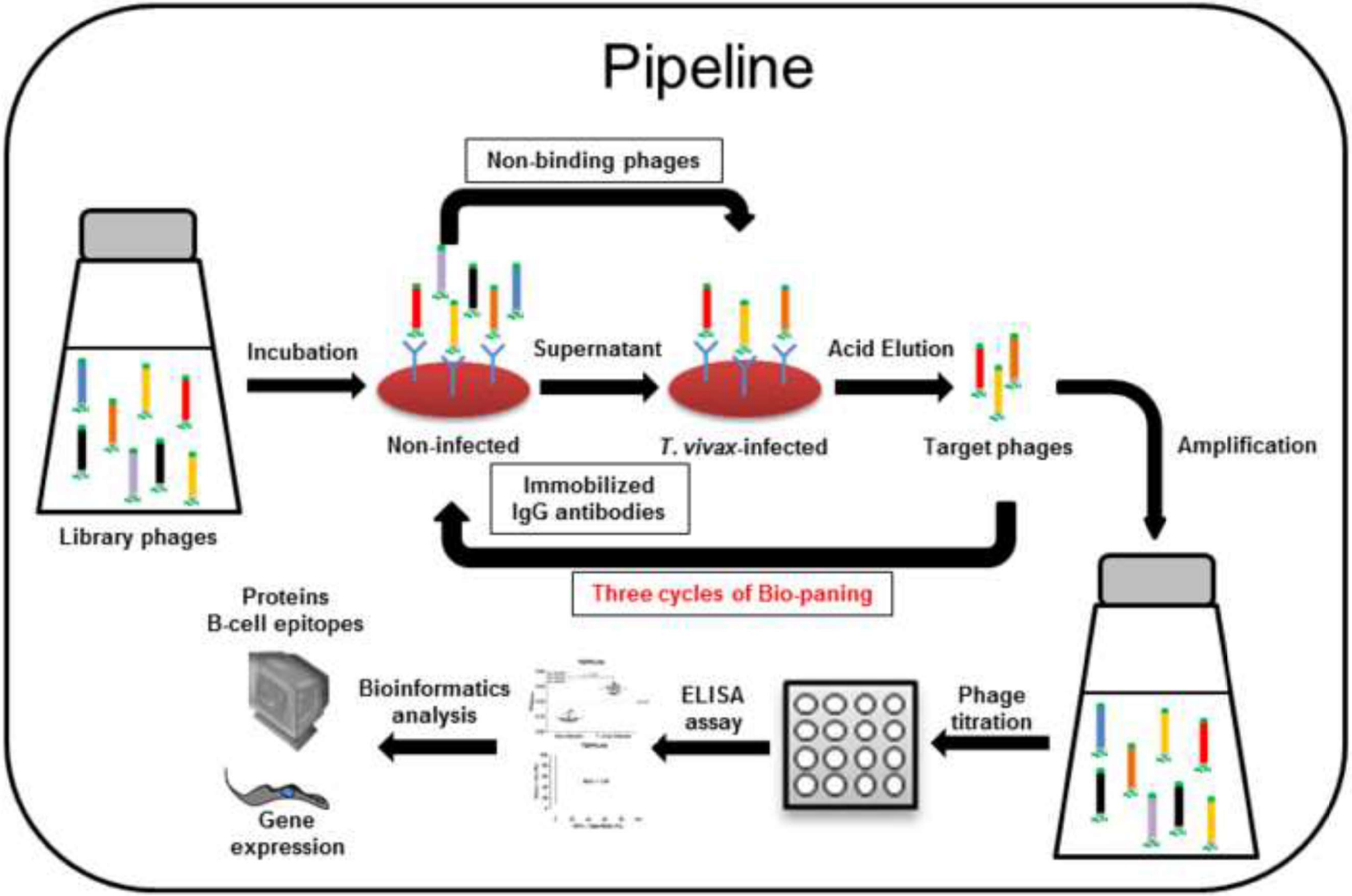

Pipeline
Non-binding phages
Incubation
Supernatant
Acid Elution
Non-infected
T. vivax-infected
Target phages
Amplification
Library phages
Immobilized
IgG antibodies
Three cycles of Bio-paning
Proteins
B-cell epitopes
Bioinformatics
analysis
ELISA
assay
Phage
titration
Gene
expression

Figure 2

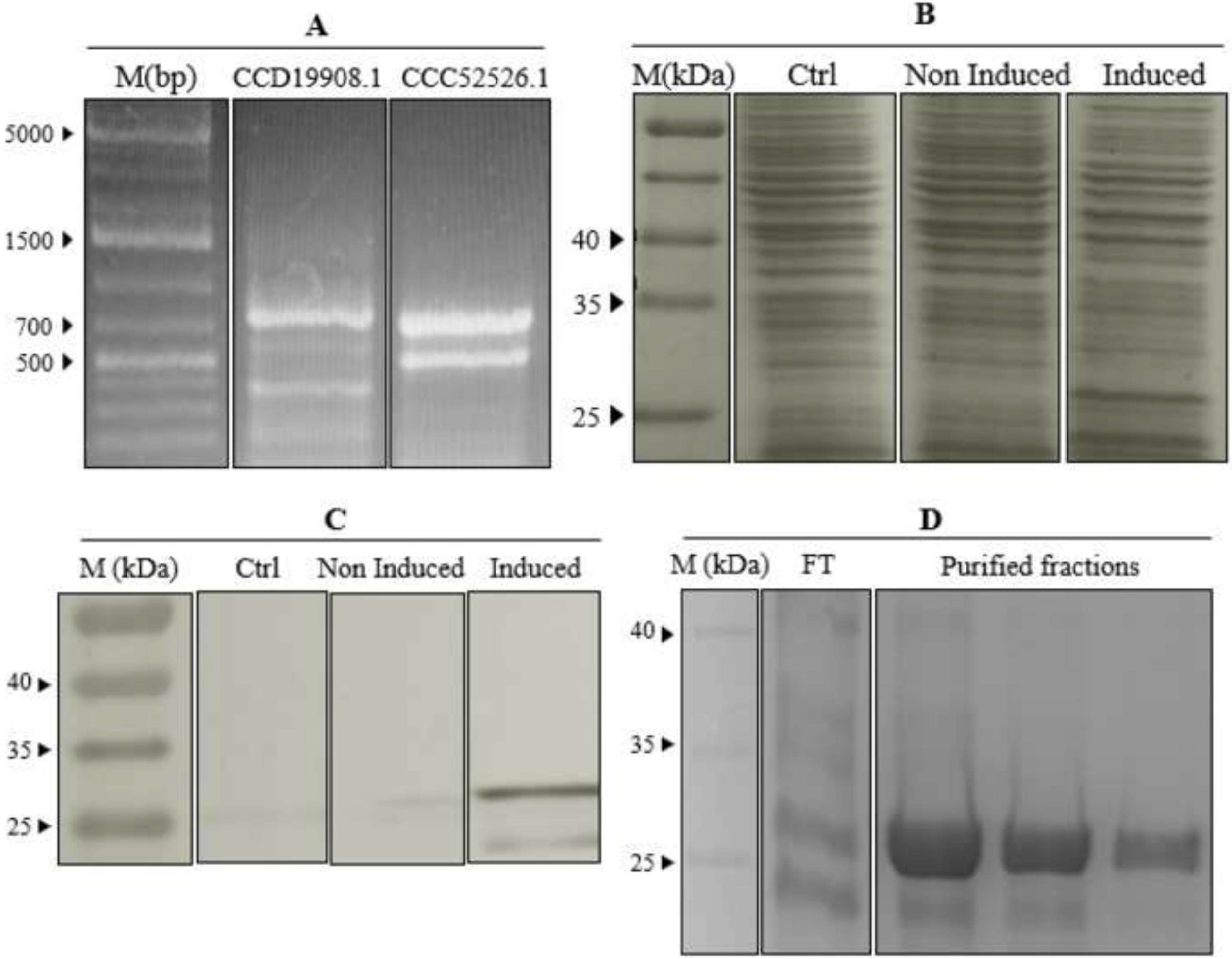
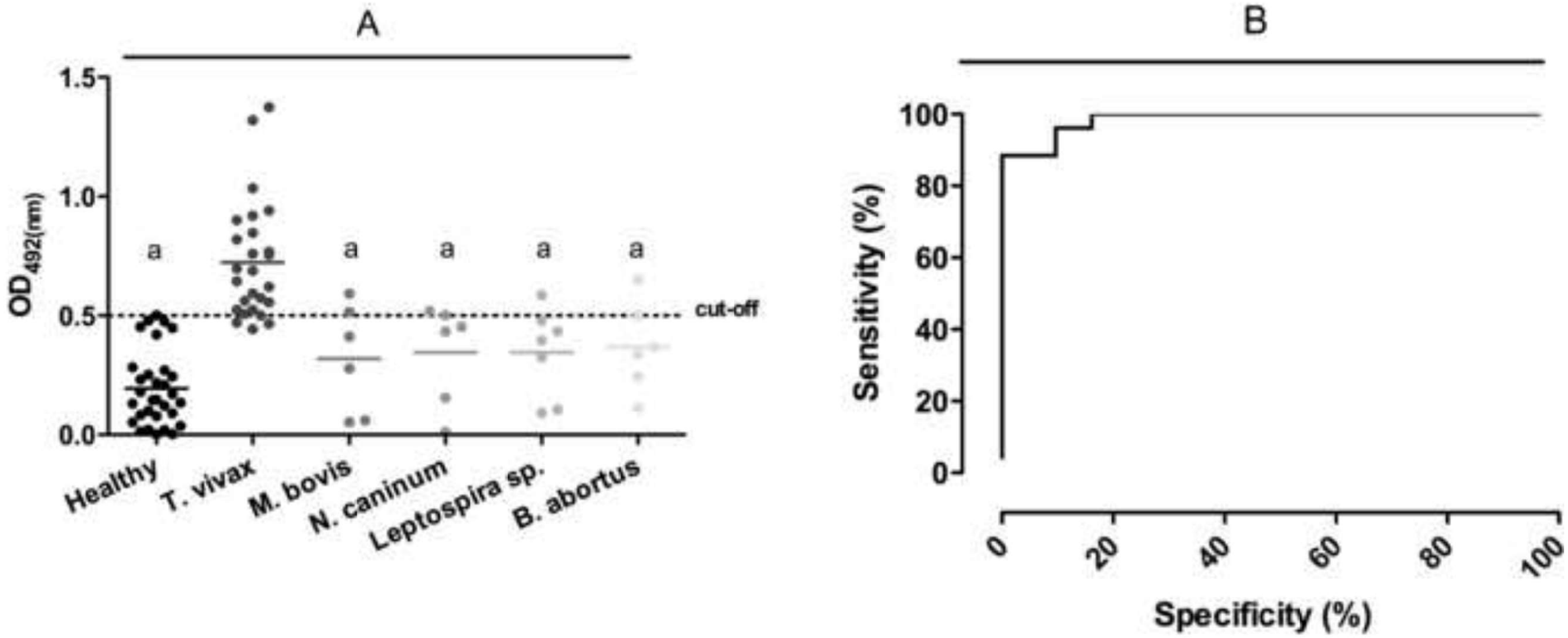

A
M(bp)
CCD19908.1
CCC52526.1
5000
1500
700
500
B
M(kDa)
Ctrl
Non Induced
Induced
40
35
25
C
M (kDa)
Ctrl
Non Induced
Induced
40
35
25
D
M (kDa)
FT
Purified fractions
40
35
25

Figure 3


A
B
$OD_{492(nm)}$
1.5
1.0
0.5
0.0
a
a
a
a
a
cut-off
Healthy
T. vivax
M. bovis
N. caninum
Leptospira sp.
B. abortus
Sensitivity (%)
100
80
60
40
20
0
0
20
40
60
80
100
Specificity (%)

CAPÍTULO II: ARTIGO PUBLICADO NA REVISTA JOURNAL OF CLINICAL MICROBIOLOGY

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Chimeric Protein Designed by Genome-Scale Immunoinformatics Enhances Serodiagnosis of Bovine Neosporosis

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ABSTRACT Neosporosis has become a concern since it is associated with abortion in cattle. Currently, *in situ* diagnosis is determined through anamnesis, evaluation of the history, and perception of the clinical signs of the herd. There is no practical and noninvasive test adapted to a large number of samples, which represents a gap for the use of new approaches that provide information about infections and the risks of herds. Here, we performed a search in the *Neospora caninum* genome by linear B-cell epitopes using immunoinformatic tools aiming to develop a chimeric protein with high potential to bind specifically to antibodies from infected cattle samples. An enzyme-linked immunosorbent assay with the new chimeric antigen was developed and tested with sera from natural field *N. caninum*-infected bovines. The cross-reactivity of the new antigen was also evaluated using sera from bovines infected by other abortive pathogens, including *Trypanosoma vivax*, *Leptospira* sp., *Mycobacterium bovis*, and *Brucella abortus*, and enzootic bovine leucosis caused by bovine leukemia virus, as well as with samples of animals infected with *Toxoplasma gondii*. The assay using the chimeric protein showed 96.6% ± 3.4% of sensitivity in comparison to healthy animal sera. Meanwhile, in relation to false-positive results provided by cross-reactivity with others pathogens, the specificity value was 97.0% ± 2.9%. In conclusion, immunoinformatic tools provide an efficient platform to build an accurate protein to diagnose bovine neosporosis based on serum samples.

KEYWORDS immunoinformatics, *Neospora caninum*, chimeric protein, serodiagnosis

Neosporosis is a parasitic disease caused by the *Neospora caninum* protozoan that affects farm animals worldwide. In the past, neosporosis was wrongly detected as toxoplasmosis due to morphological similarities with the *Toxoplasma gondii* parasite (1). Since the first report in Norwegian dogs (2), neosporosis has also become a major concern in beef and dairy cattle (3). A review study detected anti-*Neospora caninum* antibodies in dairy cattle serum samples from at least 35 countries spread across all continents (4).

Abortion is the main clinical manifestation of the disease, and it may occur in endemic or epidemic form between the third and ninth gestational months (1). However, complete understanding of determinant aspects of infection that are associated with abortion remains a challenge (5). Infected calves with no apparent sign maintain the pathogen, and they are important to disease transmission and epidemi-

Citation Pereira HS, e Almeida LT, Fernandes V, Senra RL, Fontes PP, Bittar ER, Ribon ADOB, Rotta PP, Menezes-Souza D, Bittar JFF, Mendes TADO. 2020. Chimeric protein designed by genome-scale immunoinformatics enhances serodiagnosis of bovine neosporosis. J Clin Microbiol 58:e01343-19. https://doi.org/10.1128/JCM.01343-19.

Editor Brad Fenwick, University of Tennessee at Knoxville

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Received 26 August 2019
Returned for modification 17 September 2019
Accepted 5 May 2020
Accepted manuscript posted online 13 May 2020
Published 24 June 2020

ology. These animals are clinically healthy, seropositive, and harbor the encysted parasite in several tissues. The continuous presence of dogs in farms is directly related to transmission of the parasite (6).

Currently, the promotion of epidemiologic analysis within a noninvasive examination adjusted to multiple samples is a challenge, requiring innovative mechanisms able to provide information about the routes of infections and herd risks (3, 7, 8). Farmers use anamnesis, historical evaluation of animals, and perception of clinical signs to make *in loco* diagnoses (9, 10). Nevertheless, in chronic infections, clinical signs are almost undetectable, hampering disease disclosure and enhancing its spread (6, 11). PCR is a sensitive tool to detect DNA parasites in the abortive fetus or tissue samples (12). However, *Neospora caninum* genetic variability, difficulty in perceiving the correct stages of infection, and the need to have specific equipment limits this technique (13). Serological tests that identify IgG and IgM within a few days postinfection may enable a clear perception of each infection phase (12). Among the common serological techniques, enzyme-linked immunosorbent assay (ELISA) can be easily automated to evaluate multiple samples at low cost.

Some recombinant antigens for serodiagnosis bovine neosporosis have already been described, including rNcGRA7 and rNcSAG1 (5), NcGRA7 (7), NcSRS2 (14), NcMIC10 (15), NcSAG1 (16), NcSAG4 (17), surface antigen p40 (18), and subtilisin-like serine protease 1 (19). Overall, these antigens exhibit a high potential to recognize *Neospora caninum* antibodies in infected animals, thereby differentiating healthy and toxoplasmosis-infected samples. On the other hand, these antigens do not detect cross-reactions other than abortive bovine infections, such as leptospirosis, brucellosis, and pestivirus infections, which yield a high specificity for neosporosis investigation (11). Recombinant antigens are produced on a large scale and are standardized for diagnostic tests to decrease the risks of cross-reaction (16). The use of chimeric proteins increases in an attempt to enhance diagnostic power in a combination of multiple antigens (20). In addition, synthetic antigens are easily manufactured, reaching high levels of purity in the absence of any living organism (21).

Finding an efficient, affordable, and specific method to diagnose the animal in time to avoid infection's spread among cattle is a bottleneck to controlling the disease. The production of a synthetic and highly antigenic protein that meaningfully interacts with *N. canum*-infected sera represents an improvement in diagnostic agents. The purpose of our work was to map and select antigenic epitopes based on an immunoinformatic approach to design a chimeric protein to specifically serodiagnose bovine neosporosis.

MATERIALS AND METHODS

Animals and ethics statements. Veterinary Hospital of Uberaba and Animal Science Department of Universidade Federal de Viçosa provided all serum samples used at this work. The samples were collected from Minas Gerais and Santa Catarina state, Brazil, from 2015 to 2019. Approval to use the samples was given by the Animal Experimentation Ethical Committee of the Universidade de Uberaba, Uberaba, Minas Gerais, Brazil (CEEA/UNIUBE, protocol 001/2013). The samples were collected according to the ethical principles of cattle experimentation procedures.

The bovine serum panel consisted of samples from an area of endemicity for neosporosis and other abortive cattle infectious diseases, including a neosporosis group ($n = 29$), a brucellosis group ($n = 7$), tuberculosis group ($n = 7$), trypanosomiasis group ($n = 8$), leptospirosis group ($n = 7$), and a leucosis group ($n = 5$). The neosporosis samples were from animals from Uberaba ($n = 7$, 19°44′54″S and 47°55′55″W), Viçosa ($n = 10$, 20°45′14″S and 42°52′54″W), and Lages ($n = 12$, 27°49′0″S and 50°19′35″W). The samples of others abortive infectious diseases were collected from Uberaba. A total of 8 samples of bovines infected with *T. gondii* and validated by parasite isolation from Santa Catarina state were also used (22). The samples of animals infected with *Besnoitia besnoiti* ($n = 6$) and *Sarcocystis* spp. ($n = 7$) were collected from slaughterers in Sete Lagoas (19°28′04″S and 44°14′52″W), Marília (22°13′15″S and 49°56′55″W), São José do Rio Preto (20°49′13″S and 49°22′47″W), and Petrópolis (22°30′17″S and 43°10′56″W), and the infection was confirmed by PCR using DNA extracted from brain, kidney, muscle, and lung tissues using primers previously described (23, 24). The infection by *N. caninum* was identified by using an indirect fluorescence antibody test with a cutoff of 1:100 (13) and confirmed by tissue immunohistochemistry of aborted fetuses. Identifications of animals with brucellosis and tuberculosis were performed, respectively, by using a serum (tube) agglutination test and an intradermal tuberculin test, with both followed by PCR for pathogen confirmation. Infection by *T. vivax* was confirmed by both parasitological and serological test through microscopic identification of pathogens in blood smears and detection of anti-*T. vivax* IgG by using an indirect immunofluorescence assay, respectively. Infection by

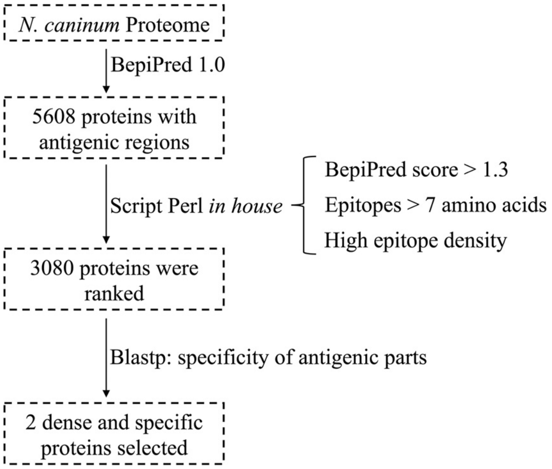

FIG 1 Workflow schematic for informatic selection of potential antigenic proteins containing a high density of linear B-cell epitopes in predicted *N. caninum* proteins from the genome sequence.

Leptospira sp. was confirmed by using a microscopic agglutination test. Bovine leucosis virus was identified by agar gel immunodiffusion. Sera of healthy animals, confirmed by negative results in parasitological tests, were also used as a control group (noninfected group, $n = 34$). Molecular techniques based on PCR were used to confirm previous histopathological tests and determine bovine monoinfection using primers previously validated and published (22–29).

Epitope identification by immunoinformatic tools. Figure 1 shows a workflow schematic that summarizes the process used for *in silico* genome-wide screening of the epitopes and design of the chimeric protein. Initially, the NCBI public database (28) provided *Neospora caninum* proteins (NCBI taxid572307) based on the genome sequence (30). A total of 21,357 proteins were submitted to the BepiPred 1.0 web server (31) to screen *Neospora caninum* linear B cell epitopes. A total of 5,608 proteins had at least a predicted epitope. Next, a Perl in-house script ranked selected proteins by (i) the average predicted BepiPred 1.0 score higher than 1.3 (the threshold value was fixed to get a confidence value higher than 96% for true epitopes), (ii) epitopes with at least seven sequentially amino acids, and (iii) the percentage of protein associated with epitopes (epitope density). Epitope density ordered more than 3,000 proteins in descendant order. Then, BLASTp (32) evaluated antigen specificity against predicted proteins of the genome sequence from pathogens belonging to the bovine environment and with symptoms similar to neosporosis, including *Trypanosoma vivax* (33), *Cryptosporidium parvum* (34), *Leptospira* spp. (35), *Mycobacterium bovis* (36), *Brucella abortus* (37), *Babesia bovis* (38), *Anaplasma marginale* (39), *Toxoplasma gondii* (40), *Sarcocystis* sp. (NCBI taxid59669), and *Besnoitia besnoiti* (NCBI taxid94643). Two proteins with high epitope density and specific to *Neospora caninum* were selected. A chimeric protein was designed by connection of the antigenic regions afforded by two chosen proteins. Flexible linkers (GSGSGS) were included within epitopes to enhance protein solubility and to prevent neighboring epitope interaction from disrupting antibody recognition (41). After the chimeric protein designed, BLASTP was used again to compare the final protein sequence to the same pathogens tested previously.

In order to produce the antigen in *Escherichia coli*, the sequence was submitted to a web server codon optimization tool (https://www.idtdna.com/CodonOpt) to increase frequent codons based on codon usage metrics and to reduce the presence of intramolecular interactions inside the mRNA molecule.

Construction and expression of chimeric protein in a bacterial system. A DNA sequence that encodes the chimeric protein was chemically synthesized in the pET28-a(+) expression system (GenScript, USA) according to the manufacturer's instructions (42). The plasmid containing the synthetic sequence was added to 50 μl of electrocompetent *E. coli* BL21 Arctic Express (DE3) cells (Agilent Technologies) by using an electroporation cuvette (Bio-Rad, Hercules, CA) and submitted to a 2.50-kV pulse using MicroPulser electroporation equipment (Bio-Rad). Bacterial transformants were grown overnight on Luria-Bertani agar plates containing 50 μg/ml of kanamycin and 20 μg/ml of gentamicin (Neoquímica, Brazil). PCRs using T7 primers were carried out to confirm all transformants before protein expression. Recombinant protein expression was induced by using 0.4 mM IPTG (isopropyl-D-thiogalactopyranoside), and the flasks were incubated with shaking for 24 h at 12°C. Sonication disrupted induced cells, and centrifugation removed all insoluble components. His-tagged chimeric protein was purified from the soluble fraction by affinity chromatography by using a His-Trap column (GE Healthcare Life Sciences). The column was washed with the buffer A containing 20 mM Na_2HPO_4, 500 mM NaCl, and 30 mM imidazole. The buffer B, containing 20 mM Na_2HPO_4, 500 mM NaCl, and 500 mM imidazole, was used to elute the protein on a gradient ranging from 0 to 100%.

Purified protein fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Initially, bacterial cells were harvested, and the pellet was resuspended with phosphate-buffered saline (PBS). Proteins were mixed in sample buffer containing

2-mercaptoethanol and heated at 95°C for 10 min. The samples were subjected to electrophoresis on a 14% polyacrylamide gel and stained using Coomassie brilliant blue R-250. The proteins were transferred to a nitrocellulose membrane, which was blocked with 5% bovine serum albumin (BSA) protein in PBS at room temperature for 1 h. The nitrocellulose membrane was washed three times in PBS buffer supplemented with 0.5% Tween 20 (PBS-T). Mouse anti-His antibody at 1:5,000 was added, followed by incubation and shaking for 2 h at room temperature. Another wash step was performed. The membrane was then incubated in peroxidase-conjugated secondary antibody at 1:15,000 for 3 h at room temperature. Finally, membrane reacted with substrate 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) and H_2O_2 for 10 min. This reaction was carried out in the dark.

The samples of purified chimeric protein were dosed by the bicinchoninic acid colorimetric method (BCA; Thermo Scientific, Waltham, MA), according to the manufacturer's recommendations, and the reading performed using SpectraMax M reader (Molecular Devices, San Jose, CA) with a wavelength of 562 nm.

ELISA and statistical analysis. ELISA was performed in MaxiSorp multiwell plates (Nunc, Roskilde, Denmark). First, the plates were coated with 1 μg of chimeric protein per well, diluted in 1 M carbonate buffer (pH 7.3). After overnight incubation at 4°C, the plates were washed five times with a washing buffer containing PBS (pH 7.2) and 0.05% Tween 20 (Sigma-Aldrich). Blocking buffer (PBS with 5% [vol/vol] BSA protein) was added, and the wells were incubated at 37°C for 1 h. An additional wash step was carried out to remove residual blocking buffer. Bovine sera were diluted 1:300 in washing buffer, and the plates were incubated at 37°C for 1 h. Another wash step was performed. Anti-bovine IgG antibody at 1:5,000, coupled with horseradish peroxidase, was then added, followed by incubation at 37°C for 1 h. OPD (*o*-phenylenediamine dihydrochloride; Sigma-Aldrich), the substrate of the reaction, was diluted in a pH 5.0 buffer containing 0.1 M citric acid, 0.2 M Na_2PO_4, and 0.02% H_2O_2, and the solution was added. The plates were kept at room temperature for 30 min in the dark. Spectrophotometric data were obtained at 492 nm using a SpectraMax M reader.

Statistical analyses were performed using GraphPad Prism (version 5.0, for Windows) and GraphPad QuickCals software. A cutoff value, based on the reactivity of sera at a lower limit of positivity, was established for optimal accuracy using the receiver operating characteristics (ROC) curve. The selected cutoff was based on the maximum sensitivity and specificity values supported by maximum-likelihood statistical metric. The performance of each test was evaluated according to the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy. A one-sample Kolmogorov-Smirnoff test was used to determine whether a variable was normally distributed. A nonparametric one-way analysis of variance (ANOVA) test was used to compare the means of absorbance between noninfected and infected groups. The degree of agreement between the ELISAs using chimeric protein with the direct diagnosis test was determined by kappa index (κ) values with 95% confidence intervals (CI) and interpreted according to the following Fleiss scale: 0.00 to 0.20, poor; 0.21 to 0.40, fair; 0.41 to 0.60, moderate; 0.61 to 0.80, good; 0.81 to 0.99, very good; and 1.00, perfect. The differences were considered statistically significant at $P < 0.001$.

RESULTS

Screening of *N. caninum* epitopes *in silico*. NCBI database provides the *Neospora caninum* proteome with 21,357 sequences. Immunoinformatic tools allowed a strict protein screening to select potential antigenic sequences, and the applied pipeline reduces the number of predict candidates for two proteins (Fig. 1). The proteins XP_003885442.1 (Fig. 2A) and XP_003884964.1 (Fig. 2B) were predicted to have more than 20% of their sequences associated with epitopes. Epitope sequences were selected according to average of BepiPred's score (Table 1) and *in silico* specificity to *N. caninum* evaluated by BLAST analysis (Table 2). In order to predict the potential specificity of epitopes, each sequence was aligned with bovine pathogens that are described in Table 2. A total of eight selected epitopes were joined using the linker sequence to produce the chimeric protein (Fig. 2C).

Validation of the expression system. The synthetic gene encodes the chimeric protein was inserted into the bacterial expression system pET-28a(+). PCR with T7 primers that anneal in pET-28a(+) vector was performed to confirm plasmid construction through amplification of a 700-bp amplicon (Fig. 3A). The amplicon size refers to the sum of a 250-bp vector amplification plus 450 bp of coding DNA.

SDS-PAGE was performed to evaluate the production of the protein by *E. coli* containing the expression system. A comparison of the protein profile was established between wild-type *E. coli* Arctic Express and the noninduced built system (Fig. 3B). It should be noted that a band at 25 kDa, even in basal expression, is associated with the expected protein, as confirmed in the purified protein lane. Western blotting was performed to validate the expression and purification of protein containing the His tag that was added to chimeric protein (Fig. 3C).

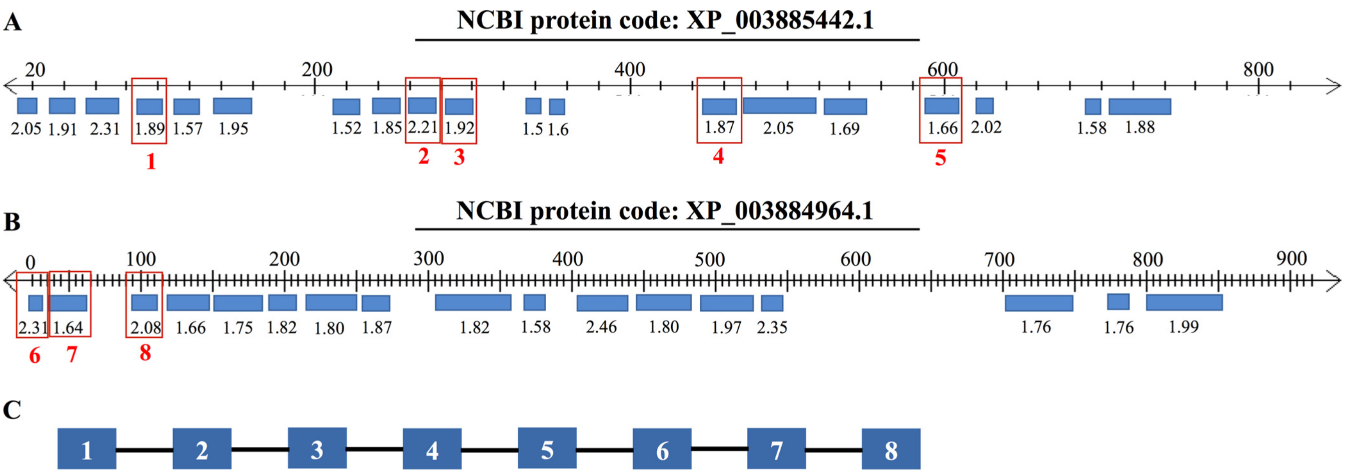

FIG 2 Epitope density of each selected protein. (A and B) Proteins XP_003885442.1 and XP_003884964.1, respectively. Blue boxes represent the predicted epitopes on protein sequence, and the average BepiPred score for each peptide is shown below the blue box. Red outlines indicate selected epitopes, and the red numbers indicate the order of peptides in the chimera protein. (C) Schematic showing the chimera sequence, with the numbered peptides representing each selected epitope from the original sequence. Black lines represent the GSGSGS linkers added between each epitope.

Evaluation of chimeric protein serodiagnostic potential. An ELISA was performed to validate and characterize protein reactivity against field-infected animal sera. The serum antibody form *N. caninum*-infected bovine showed strong reactivity with the chimeric protein (Fig. 4A). Statistical analysis was performed using an optimized cutoff value of 1.089 (Table 3). The chimeric protein showed 96.6% sensitivity compared to healthy animal sera. Meanwhile, a specificity value of 97.0% was calculated based on the relation to false-positive reactivity afforded to bovine pathogens and noninfected sera. Furthermore, 96.5 and 96.9%, respectively, are the probabilities that the disease is present when the test is positive and not present when the test is negative. A kappa index of 0.935 represents an almost perfect agreement within results provided for histopathological and molecular tests compared to those obtained using chimeric protein. A nonparametric one-way ANOVA test with a P value of <0.001 compared to neosporosis and settled groups gave statistical support to the data. The ROC curve showed that the selected threshold yielded high specificity and sensitivity values (Fig. 4B).

DISCUSSION

In summary, the present study applied immunoinformatic tools to screen antigenic proteins in a predicted proteome of the *N. caninum* genome sequence. Based on this screen, we built a chimeric protein to serodiagnose bovine neosporosis by combining the predicted antigenic regions with high potential specificity. For this, the sequences of 21,357 proteins were evaluated to provide eight epitopes as a cast for the synthetic molecule. Our data suggest that an *in vitro*-assembled protein could be easily expressed

TABLE 1 Selected potential epitopes of *N. caninum* proteins to compose the chimeric protein

NCBI protein code	Epitope	Sequence	Length (aa)[a]	BepiPred score
XP_003885442.1	1	YSPPGAPAGQNME	13	1.89
	2	SRDSPPSPAGGAT	13	2.21
	3	NPRRGHPTGEPRG	13	1.92
	4	GRPPRGGYEPHRGRPEPEAM	20	1.87
	5	SLGPSGPSLSSPE	13	1.66
XP_003884964.1	6	MGPPPTAASGA	11	2.31
	7	SSGVPSSANASPSSSATA	18	1.64
	8	PPPGRFTGNPPASPGGRQ	18	2.08

[a] aa, amino acids.

TABLE 2 Similarity of each selected *N. caninum* epitope with others bovine pathogens accessed by BLASTp

	Similarity (%)									
Epitope	*Toxoplasma gondii*	*Trypanosoma vivax*	*Cryptosporidium parvum*	*Leptospira* spp.	*Mycobacterium bovis*	*Brucella abortus*	*Babesia bovis*	*Anaplasma marginale*	*Sarcocystis* spp.	*Besnoitia besnoiti*
1	53.8	53.8	53.8	61.5	69.2	53.8	53.8	46.1	22.5	58.3
2	76.9	46	61.5	23	46	46	48.4	46	30.7	83.3
3	61.5	46	46	53.8	46	0	53.8	30.7	0	70
4	60	40	35	40	0	40	30	25	20	61.1
5	61.5	61.5	61.5	61.5	53.8	61.5	53.8	53.8	30.3	90
6	100	54.5	63.6	63.6	54.5	63.6	36.3	54.5	36	72.7
7	67	55.5	55.5	50	55.5	44.4	27.7	11	27.5	76.9
8	67	22	38.8	55.5	27.7	22	27.7	0	21.8	58.8

in a bacterial system, and it can highly discriminate *N. caninum*-infected sera from other sera.

Mapping and identification of specific linear B-cell epitopes that bind to antibodies induced by pathogens represents a challenging step in immunological investigation. Epitope mapping is quite useful in vaccine development, antibody production, and the rational design of therapeutic protein and provides clues to improve peptide immunogenicity (21). Experimental B-cell epitope determination is costly and difficult work that allows an accurate investigation of stagger informatic approaches (43). Initially, proteome-wide screening required *N. caninum*-specific and antigenic proteins with epitope regions higher than 10% of total sequence length (Fig. 2). Determination of linear B-cell epitopes represents the first step in mapping antigens against specific pathogens, and it should be based on reliable and simple bioinformatics tools to rating in genomic scale (31). The BepiPred 1.0 web server made a refined search employing a threshold of 1.3, which supports the specificity (96%) of prediction regarding sensitivity, increasing the chance of being a true epitope. The amino acid linear segment may vary from 2 to 9 when the entire sequence is considered, and the epitope could reach more than 17 amino acids when nonepitopes are considered (44). Hence, selected epitopes are sized within 11 to 20 amino acids and have a medium score higher than 1.3 (Table 1). The percentages of identity values indicate an average that is smaller than 70% versus other bovine pathogens, with higher diversity values for epitopes 4 and 8 (Table 2). As an example of the importance of the joined epitope sequence, a study fragmented antigenic elements of the NcGRA7 protein and detected a decrease in antibody recognition (7). Aiming to construct a robust and all-antigenic molecule, the GSGSGS linker combined chosen epitopes to form a chimeric protein.

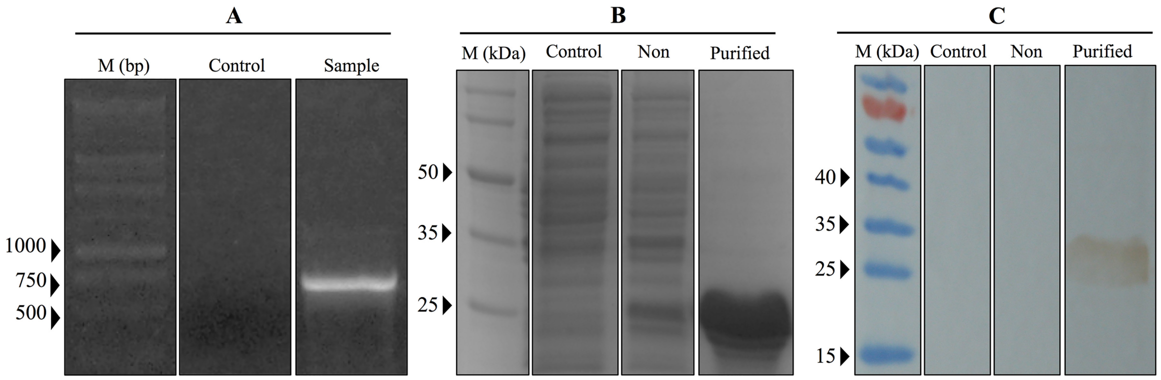

FIG 3 Confirmation of chimera expression in *E. coli*. (A) PCR confirmation of *E. coli* Arctic Express containing the chimeric gene transformation protocol with DNA plasmid. (B) SDS-PAGE protein profile in a wild-type system (Control), a non-IPTG-induced system (Non), and after protein purification (Purified). (C) Western blot analysis with anti-His antibody was performed to evaluate the expression of chimeric protein containing His tag in a wild-type system (Control), a non-IPTG-induced system (Non), and after protein purification (Purified).

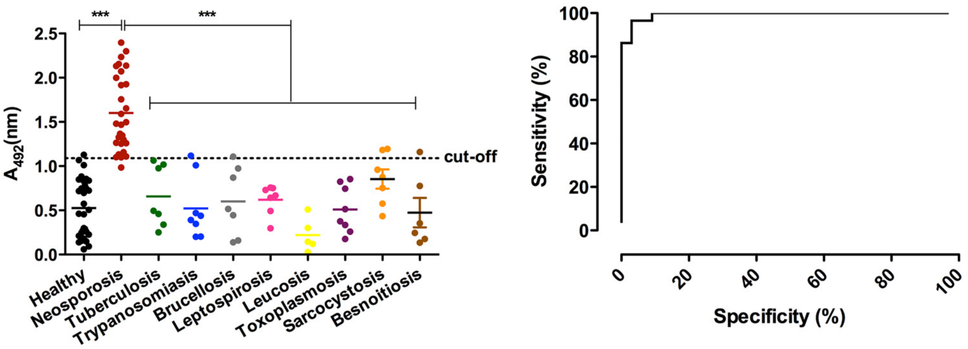

FIG 4 Evaluation of the diagnostic potential of chimeric proteins. (A) ELISA reactivity of healthy samples (*n* = 34, black circles), neosporosis samples (*n* = 29, red circles), tuberculosis samples (*n* = 7, green circles), trypanosomiasis samples (*n* = 8, blue circles), brucellosis samples (*n* = 7, gray circles), leptospirosis samples (*n* = 7, pink circles), leucosis samples (*n* = 5, yellow circles), toxoplasmosis samples (*n* = 8, purple circles), sarcocystosis samples (*n* = 7, orange circles), and besnoitiosis samples (*n* = 6, brown circles) against the chimeric antigen. The neosporosis samples are statistically distinct from the other values with a *P* of <0.05 as determined in a one-way ANOVA analysis. (B) ROC curve showing the relationship between assay sensitivity and specificity.

Glycine-rich linkers are flexible, preserve individual domain function, afford structural stability, and smooth protein folding (41).

The SDS-PAGE protein pattern ensures that the concentrations of all tested samples were equivalent (Fig. 3B). In addition, should be noted that in a 25 kDa that there was a differential pattern among control and noninduced lanes which indicates a system basal expression. Also, it should be noted in the purified lane that the 25-kDa protein concentration suggests a high protein purification level sufficient for ELISA. In Western blot analyses, an anti-His antibody recognized the purified chimeric protein sample instead of *E. coli* Arctic Express control cells and noninduced lanes (Fig. 3C). In general, these results imply that the bacterial expression system was effective in chimeric protein expression with a preserved sequence considering that the molecule was marked in expected size. Antigen prediction was looking for linear epitopes, so the preserved protein sequence increases the affinity with antibodies anti-*N. caninum* from infected samples.

ELISA is a high-throughput, fast, and cost-effective method that can be used to investigate a multitude of samples in fewer reactions and has been well characterized in serological diagnosis (45). The indirect fluorescent antibody test is a reference technique used to diagnose neosporosis, although it is expensive, laborious, and less accurate than ELISA (14). An indirect ELISA with chimeric protein as the antigen was performed to measure diagnostic potential in serum samples (Fig. 4A). Multiepitope or chimeric proteins demonstrate better performance in serodiagnosis compared to isolated antigens (46). The results show that the chimeric protein associates strongly with infected animals anti-*N. caninum* IgG antibody. The generated data yielded an ROC curve that presents the relationship between specificity and sensitivity (Fig. 4B). For a diagnostic approach, the chosen cutoff value provided 96.5% sensitivity, picking 28 of

TABLE 3 Statistical analysis of ELISA results

Parameter[a]	Result
% (95% CI)	
Sensitivity	96.5 (±3.3)
Specificity	92.9 (±5.0)
PPV	94.5 (±2.9)
NPV	98.7 (±1.1)
Accuracy	94.3 (±4.1)
Kappa index	0.91 (±0.08)
P	$2.71e^{-14}$

[a] PPV, positive predictive value; NPV, negative predictive value.

29 true-positive sera. On the other hand, the cutoff value differentiated 74 to 80 false-negative results (92.9% specificity) (Table 3). By comparing the sensitivity and specificity parameters to the average diagnostic accuracy, we found that the chimeric protein afforded equivalent or better values than those presented in the literature (5, 7, 14–19). Moreover, the chimeric protein can discriminate infected samples from abortive diseases such as brucellosis, leptospirosis, and trypanosomiasis, which were not tested in previous articles, as well as from phylogenetically related pathogens, including *T. gondii*, *Sarcocystis* spp., and *Besnoitia besnoiti*. ELISA achieved numbers that were all predicted in each epitope immunoinformatic study (Table 2). It should be noted that after combining epitope pieces, the predicted sequence differences were enhanced. For further analysis, testing sera collected from non-South American animals would broaden the genetic diversity of *N. caninum* strains, increasing the diagnostic power of the chimeric protein worldwide.

N. caninum infections are commonly confused with those caused by *T. gondii* in cross-reactions (7). Since 1986, *T. gondii* has been reported as a nonabortive protozoan in cattle because the pathogen is quickly eliminated from animal tissue (47). More recently, the presence of *T. gondii* in aborted fetuses is not confirmed, and parasite identification in naturally infected cattle is rare (48). In a Brazilian case report, the incidence of toxoplasmosis in sheep was 80 times higher than in infected cattle (49). A United Kingdom research group studied the prevalence of *T. gondii* naturally infected cattle and found that only 1.79%, in a group of 305 animals, carried the disease (50). Another study indicates the low tissue presence of viable *T. gondii* parasite, even in experimentally infected animals (51). Therefore, bovine toxoplasmosis is a rare phenomenon in naturally infected cattle, and usually the infection is not associated with abortion. However, Table 2 shows differences within predicted epitopes and *T. gondii* proteins, which implies different intensity reactions with toxoplasmosis sera, as confirmed in other bovine infections. Nevertheless, we performed PCR screening of the samples to confirm that the infection was caused by *N. caninum*, and none of them were amplified to a *T. gondii*-specific primer. In addition, we included rare samples of *T. gondii*-infected cattle that showed no cross-reaction with the antigen. There have been many studies that indicate similarities within infections induced by *N. caninum*, *Sarcocystis* spp., and *Besnoitia besnoiti* (47, 48, 52, 53). Protein sequence similarities were assessed among eight selected epitopes with *Sarcocystis* spp. (taxid 59669) and the *Besnoitia besnoiti* (taxid 94643) proteome utilizing BLASTP tool. Lower identity values seen in every epitope concerning *Sarcocystis* sp. suggests that there is no cross-reaction with this pathogen, which was confirmed by ELISA with sera from animals infected with this pathogen. On the other hand, these epitopes have a high identity compared to the *B. besnoiti* proteome. However, ELISA using a small set of samples ($n = 6$) demonstrated low cross-reactivity with the chimeric antigen. To date, a single study has been intended to evaluate the frequency of anti-*B. besnoiti* antibodies in ruminants throughout South America (49). According to the authors of that study, the low titers detected do not match those already described in Africa and Europe, and more investigations are needed to characterize the disease epidemiology and immunological response to infection in Brazilian territory. It is also worth mentioning that reports observed in the literature indicate that infections caused by this organism lead to biochemical alterations in the blood (50), a drop in milk production (51), and reproductive failures (54), with no reports indicating the involvement of bovine besnoitiosis to abortion.

Statistical analysis was performed to better understand the collected data. The positive and negative predictive values indicate that the detected individual is correctly grouped. Consequently, the accuracy in classification of samples shows a significative value with a low error margin (Table 3). The kappa index suggests an agreement among gold standard techniques, such as molecular and histopathological tests and serological assay. The closer the kappa index is to 1, the higher the test agreement. According to Landis and Koch (55), a value of 0.91 indicates an almost perfect correlation.

To our knowledge, no study has evaluated chimeric protein potential in bovine neosporosis diagnosis. In the present study, immunoinformatic tools provided valuable information in antigenic molecule functional screening, allowing us to develop a synthetic protein that was effective in definitive disease diagnosis. Moreover, the applied procedure facilitates the construction of powerful molecules to recognize a diversity of diseases.

ACKNOWLEDGMENTS

We thank the Diretoria de Tecnologia da Informação from the Universidade Federal de Viçosa for providing access to the computer cluster Jupiter for informatic analysis.

We acknowledge financial support from the Coordenacão de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) provided as student and researcher fellowships during project execution.

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CAPÍTULO III: SELEÇÃO DE APÂMEROS DE DNA ESPECÍFICOS PARA PROTEÍNA QUIMÉRICA DE *NEOSPORA CANINUM*

Seleção de aptâmeros de DNA específicos para proteína quimérica de *Neospora caninum*

Introdução

Neosporose bovina é uma doença causada pelo protozoário do filo Apicomplexa *Neospora caninum* que, por sua vez, é um parasita intracelular obrigatório comumente associado a problemas reprodutivos em animais em todo o mundo (1). Ao longo dos anos, tem-se visto esforços crescentes na busca por um tratamento eficaz para a neosporose. Pesquisadores descobriram que o Triclosan atua como um agente inibidor in vitro e in vivo da enzima carreadora enoyl acyl redutase, consequentemente limitando o crescimento do protozoário (2). Um outro estudo desenvolveu um anticorpo monoclonal baseado na proteína 14-3-3 de *Neospora caninum* como um estimulante do sistema imune a partir da expressão de citocinas (1). No entanto, nenhum tratamento eficaz ou vacina estão disponíveis no mercado (3). Por outro lado, a contaminação causada por este protozoário é normalmente acompanhada de uma sintomatologia pouco específica, dificultando a compreensão precisa dos sinais clínicos em animais (4). Uma técnica singular que se aplique ao diagnóstico imediato da infecção auxiliará os produtores no manejo e aplicação das medidas profiláticas adequadas.

Historicamente, o diagnóstico in situ da neosporose é estabelecido a partir da percepção dos sinais clínicos, avaliação do histórico do animal e anamnese (5, 6). Entretanto, ao longo dos anos, vários estudos buscaram aprimorar a detecção sorológica e molecular do protozoário e/ou seus componentes. Tanto a Reação em Cadeia da Polimerase (PCR, do inglês *Polymerase Chain Reaction*) convencional quanto em tempo real são técnicas refinadas para a detecção do material genético do parasito em diferentes tecidos, como cérebro, coração e placenta (7). Já se é sabido que os anticorpos gerados a partir da infecção por Neospora caninum em bovinos são encontrados em diversos fluídos corporais, dentre eles, em ordem de frequência, soro, leite, secreção vaginal e saliva (8). Adicionalmente, a identificação sorológica frequentemente busca alvos antigênicos da superfície celular de *Neospora caninum* (NcSAG) ou proteínas cujas sequencias estejam relacionadas às de superfície (NcSRS) (9). A proteína 10 do micronema (NcMIC10) e a proteína granular 7 (NcGRA7) também são alvos de estudo para atuação como biomarcadores na detecção da doença (10, 11). Todavia, estes procedimentos testam os antígenos contra soros positivos, negativos e infectados por toxoplasmose para estabelecer um diagnóstico seguro. Logo, estes estudos desconsideram uma série de patógenos geneticamente similares ou cuja sintomatologia ao infectar os ruminantes se assemelhem as apresentadas pelo quadro de neosporose. Apesar do potencial de automatização dos métodos sorológicos que permitem triagem de um elevado número de amostras, os testes sorológicos possuem como desvantagem as aplicações indiretas que buscam detectar a resposta através de anticorpos, ao invés de moléculas do patógeno, que continuam altos mesmo após cura do animal.

Em nosso recente trabalho (Capítulo 2), construímos uma proteína quimérica desenhada por ferramentas de imunoinformática, em uma escala genômica, para diagnóstico da neosporose bovina. Para tanto, executamos uma triagem em busca de epítopos lineares de células B e os confrontamos com as sequências de microrganismos filogeneticamente próximos a *Neospora caninum* e também com aqueles causadores de aborto em bovinos. Além do mais, a proteína quimérica foi testada em imunoensaios contra os soros dos animais infectados com estes patógenos, apresentando sensibilidade de 96,5% e especificidade de 92,9%.

Por outro lado, aptâmeros têm sido utilizados como ferramentas de diagnóstico em ensaios imunológicos semelhantes ao ELISA, em dispositivos para ensaios de fluxo lateral e na conjugação com nanomateriais (12). Aptâmeros são pequenos oligonucleotídeos de DNA ou RNA de cadeia simples, altamente estruturados, que apresentam elevada afinidade e especificidade na interação com moléculas alvo. A seleção de aptâmeros ocorre por meio de um processo aleatório, conhecido como SELEX, em ciclos iterativos de incubação do pool de nucleotídeos com o alvo, conforme mostrado na Figura 1. Os ciclos de seleção são geralmente divididos em quatro estágios: incubação, seleção, eluição e amplificação. Os biosensores baseados em aptâmeros, aptasensores, podem ser conjugados com uma ampla diversidade de moléculas, sem modificar sua atividade e afinidade com o alvo (13). Transdutores de sinal comumente conjugados com os aptasensores incluem a adição de moléculas repórteres que emitem sinais colorimétricos, eletroquímicos ou fluorescentes (14). Além do mais, nanomateriais como nanopartículas de ouro, prata, platina, óxido de ferro ou nanotubos de carbono, possuem protocolos de conjugação a aptâmeros e proteínas bem caracterizados na literatura (14, 15). Embora bem caracterizados, os aptasensores possuem uma imensidão de aplicações ainda não exploradas.

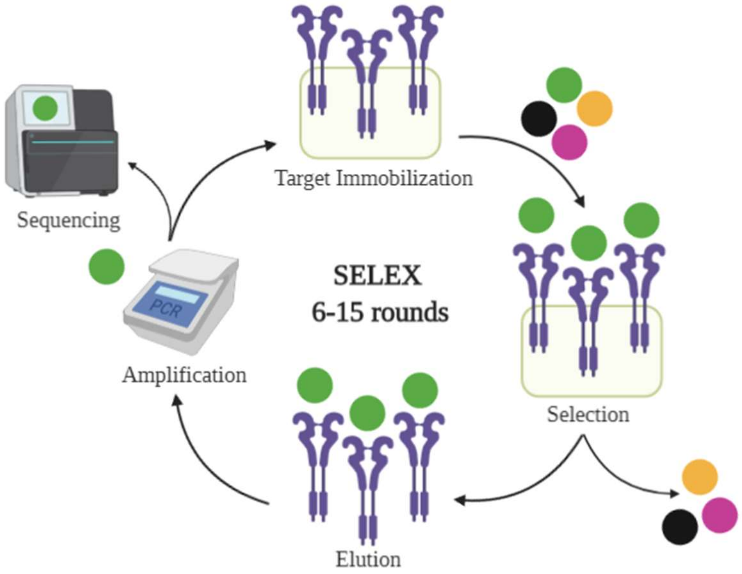

Figura 1 - Representação esquemática das etapas de seleção de aptâmeros para alvos proteicos imobilizados em membrana de nitrocelulose. Os círculos coloridos representam biblioteca de oligonucleotídeos com diferentes sequências.

Diante deste cenário, busca-se com este trabalho estabelecer um protocolo para seleção de aptâmeros de DNA que possam atuar na detecção de proteínas específicas, imobilizadas em membranas de nitrocelulose. Para tanto, a proteína quimérica desenhada a partir de epítopos lineares de células B servirá como ponto de partida para seleção das sondas de aptâmeros pelo método de SELEX.

Metodologia

- Desenho, síntese e expressão da proteína quimérica

Toda estratégia utilizada para o desenho, síntese e expressão da proteína quimérica em sistema bacteriano está descrita detalhadamente no Capítulo 1.

- Seleção de aptâmeros

A estratégia empregada para seleção dos aptâmeros foi desenhada a partir do protocolo de SELEX de proteínas em membranas de nitrocelulose já descrito na literatura (16). Inicialmente, uma biblioteca randômica de oligonucleotídeos de DNA fita simples foi adquirida junto a empresa ThermoFisher (ThermoFisher, Waltham, MA), flanqueada pelos primers *foward* 5' TGC CAC CTG ACG TCT AAG AA 3' e *reverse* 5´ GCT CAC TCA AAG GCG GTA A 3', contendo uma região variável e randômica de 40 nucleotídeos (N40). Em cada round de seleção, foi executada a seleção positiva para enriquecimento dos aptâmeros ligantes a proteína quimérica. Os parâmetros para seleção de cada round estão detalhados na Tabela 1. A proteína foi incubada em tampão de ligação (20 mM HEPES, 0,15 mM NaCl, 2 mM $CaCl_2$) a temperatura ambiente no tempo determinado para cada round. Após, esta solução contendo proteínas e aptâmeros foi incubada sob as mesmas condições anteriores em contato com a membrana de nitrocelulose. Em seguida, o complexo formado entre membrana, proteína e aptâmeros foi separado a partir da imersão em solução de fenol/clorofórmio/álcool isoamílico e vortexado por 1 minuto. Um outro volume de H_2O foi adicionado à solução, que foi novamente vortexada e centrifugada a 17.000 x g por 10 minutos para separação em duas fases. Após, a fase aquosa (superior) foi coletada e misturada em um volume igual de clorofórmio para remoção de resíduos de fenol remanescentes em solução. Os microtubos foram então vortexado por 1 minuto e centrifugados a 17.000 x g por 10 minutos. Novamente a fração aquosa foi coletada e o DNA presente em solução foi precipitado a partir da adição de 5 µL de acrilamida linear, 1/10 do volume de acetato de amônio 10M e 2,5 volumes de etanol 100%. Esta solução foi, então, misturada e incubada por 16h a temperatura de – 20 ºC. Após este intervalo, os microtubos contendo a solução foram centrifugados a 17.000 x g por 10 minutos, o sobrenadante foi removido e o *pellet* será lavado com 1 ml de etanol 95%. Novamente, a solução foi centrifugada a 4ºC sob rotação de 17.000 x g por 10 minutos, o sobrenadante removido e pellet foi seco em temperatura ambiente. Em seguida, os aptâmeros precipitados foram recuperados em solução de 25 µL de H_2O e enriquecidos pela PCR. As condições de amplificação serão: 95 °C por 5 minutos, 30 ciclos de 95 °C por 30 segundos, 50 °C por 30 segundos e 72 °C por 30 segundos; e extensão final de 72 °C por 3 minutos. As sequências amplificadas em cada *round* serviram como molde para a seleção no round seguinte. Vale ressaltar que, a partir do *round* 2, todas as soluções de DNA foram previamente aquecidas a 95 °C por 5 minutos e rapidamente resfriadas no gelo por 5 minutos para que assumam a conformação estrutural a partir de uma fita simples.

Tabela 1 – Protocolo de seleção de aptâmeros de DNA em membrana de nitrocelulose.

Round	DNA (pmol)	Proteína (pmol)	Volume de reação	Tempo de ligação	Lavagem (10 mL)
1	1000	200	50 mL	30 min em solução 30 min em membrana	3 x 5 min
2	1000	100	10 mL	10 min em solução	3 x 5 min

				10 min em membrana	
3	1000	100	10 mL	5 min em solução 5 min em membrana	3 x 10 min
4	1000	50	10 mL	5 min em solução 5 min em membrana	3 x 10 min
5	2000	50	10 mL	5 min em solução 5 min em membrana	3 x 10 min
6	2000	25	10 mL	5 min em solução 5 min em membrana	3 x 15 min
7	2000	25	5 mL	5 min em solução 5 min em membrana	3 x 30 min

- Caracterização dos aptâmeros

A sequência dos oligonucleotídeos selecionados como aptâmeros foram identificas através do sequenciamento de Sanger. Para tanto, os aptâmeros selecionados no sétimo round de seleção foram amplificados sob as seguintes condições: 95 °C por 5 minutos, 30 ciclos de 95 °C por 30 segundos, 50 °C por 30 segundos e 72 °C por 30 segundos; e extensão final de 72 °C por 3 minutos. O produto de PCR foi ligado em vetor pGEM e deixado overnight à 4 ºC. Em seguida, os plasmídeos contendo os aptâmeros entre as regiões M13 foi transformado por choque térmico em *Escherichia coli* XL1 *blue* para multiplicação dos clones. Os transformantes foram plaqueados em meio sólido e 20 colônias foram inoculadas em meio líquido, tiveram seu plasmídeo extraído e amplificados com primers M13 para confirmação da inserção dos aptâmeros no vetor. Após, 12 colônias foram confirmadas por PCR, seus plasmídeos foram então extraídos e enviados para a empresa ACTgene Análises Moleculares (Rio Grande do Sul, Brasil) para sequenciamento de Sanger, com os primers M13F (GTAAAACGACGGCCAG) e M13R (TGCCACCTGACGTCTAAGAA).

O software Chromas 2.6.6 foi utilizado para análise do cromatograma produzido pelo sequenciamento de Sanger. Para trimagem dos picos de baixo qualidade, foi utilizado o parâmetro *phred* 20 (99% de acurácia). Após a trimagem, as sequências *forward* e *reverse* foram montadas pelo servidor CAP3 (http://doua.prabi.fr/software/cap3) para geração do *contig*. A sequência do *contig* foi então avaliada e alinhada no servidor Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) para que se encontre as regiões flanqueadas pelos primers da biblioteca de aptâmeros.

Resultados e discussão

Todas as etapas de construção, síntese, expressão e purificação da proteína foram indicadas na Figura 3, do Capítulo 2. Até o presente momento, a proteína quimérica foi imobilizada em membrana de nitrocelulose e as etapas de seleção dos aptâmeros foram executas em sete *rounds*, de acordo com os critérios detalhados na Tabela 1. Como controle de cada ciclo, os aptâmeros eluídos foram amplificados com *primers* específicos da biblioteca, conforme demonstrado na Figura 2. Até o sétimo *round* de seleção, foi possível precipitar oligonucleotídeos que interagiram com a proteína selecionada com um aumento gradativo dos parâmetros de seleção.

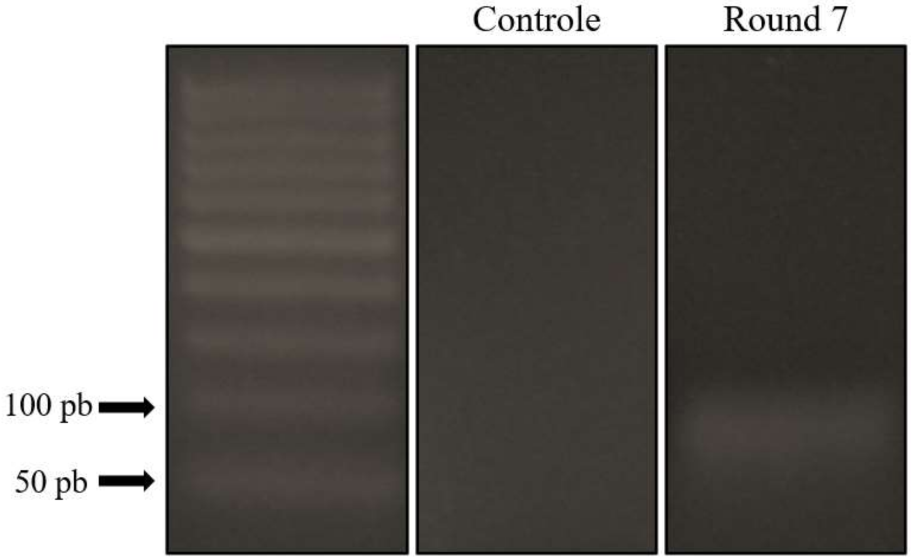

Figura 2 – PCR da amplificação dos aptâmeros eluídos no sétimo *round* de seleção. No controle, foi utilizado o mesmo *mix* de amplificação, sem a adição do *template* de DNA. Pode-se notar que houve uma amplificação em aproximadamente 80 pares de base, o tamanho esperado a partir do desenho da biblioteca.

Entre os 12 plasmídeos enviados para sequenciamento, foram recuperadas 5 sequências consenso com regiões de nucleotídeos bem definidas e de boa qualidade entre os primers que flanqueiam a biblioteca (Tabela 2). As sequências foram avaliadas em conjunto a fim de buscar uma região conservada entre os aptâmeros encontrados. Porém, nenhuma sobreposição que caracterizasse uma evolução gradativa dos rounds pode ser vista através do sequenciamento de Sanger. Neste sentido, entende-se que o método de sequenciamento não seja o adequado para analisar a evolução das sequências nem a diversidade dentro de um pool de oligonucleotídeos. Para envio das amostras, é necessário cloná-las e selecionar os clones que amplificaram com primers específicos, este fato limita o rastreio de diferentes sondas tanto em termos financeiros quanto, devido ao preço individual do sequenciamento, quanto em termos de diversidade de sequência. Por isso, acredita-se que o mais recomendando nesta situação seria um

Sequenciamento de Nova Geração (NGS, do inglês *Next-generation Sequencing*) pois fornece uma análise mais minuciosa e precisa acerca da sequência dos componentes que interagiram com a proteína alvo.

Tabela 2 – Sequência de aptâmeros recuperadas a partir do sequenciamento de Sanger.

Aptâmero	**Sequência (5'-3')**
Apta6.2	CAATAACGACGTAGTGTGGTTAGCATGCATANCTTTGCAGAAT
Apta6.5	CTTATTTCTGCGGGAAGAGATTTGTATAGTGATATGAAATAT
Apta7.1	CGTGATGCGCAGAAAAGAGCATCTTTGTAATTGTTTGTCCAT
Apta7.5	GTGAGCCTTGTGACGTGGGACATAAATAATTTGTAAAATTAT
Apta7.8	TGGGAGTTAGTCGTAAACGCGCCTTTCTCAGTAATAACGGAT

No entanto, a partir das sequências obtidas na Tabela 2, foi possível fazer a predição da estrutura secundária de menor energia destes aptâmeros selecionados (Figura 3). Pode-se notar que todos tem energia negativa, o que indica que estas estruturas podem ser formadas naturalmente em sistemas biológicos. Além disso, vale destacar que, em geral, as estruturas preditas não são homogêneas, assumindo diferentes dobramentos. Isso é facilmente explicado pela diversidade de sequência destes oligonucleotídeos, pois: maior conservação de sequência, maior conservação estrutural. Mesmo assim, é possível apontar que em todos os dobramentos preditos é visto um pequeno *loop* de aproximadamente 28 nucleotídeos (destacado por um círculo amarelo) no meio ou no final do domínio estrutural. Nas estruturas Apta6.2, Apta6.5 e Apta7.8 há formação de *hairpin* e um *loop* interno, enquanto que em Apta7.5 e Apta7.1 o domínio formado é do tipo *stem-loop*, um tipo de *hairpin*. Estes domínios mantidos, mesmo em sequências pouco conservadas, indicam que esta região pode ser responsável pela interação com algum sítio da proteína quimérica. Se tratando de uma proteína sintética, fica difícil assumir ou predizer quais sítios estão disponíveis para interação. Portanto, é necessário que se explore melhor os dados gerados a partir de um volume maior de aptâmeros selecionados para então decidir: ora pelo aumento do número de *rounds* para que se continue restringindo a uma sequência menos diversa e mais conservada; ora decidir que estas sequências, dentre todo o *pool* inserido no início da seleção, são as que interagem mais fortemente com esta proteína através destes domínios de *loop*.

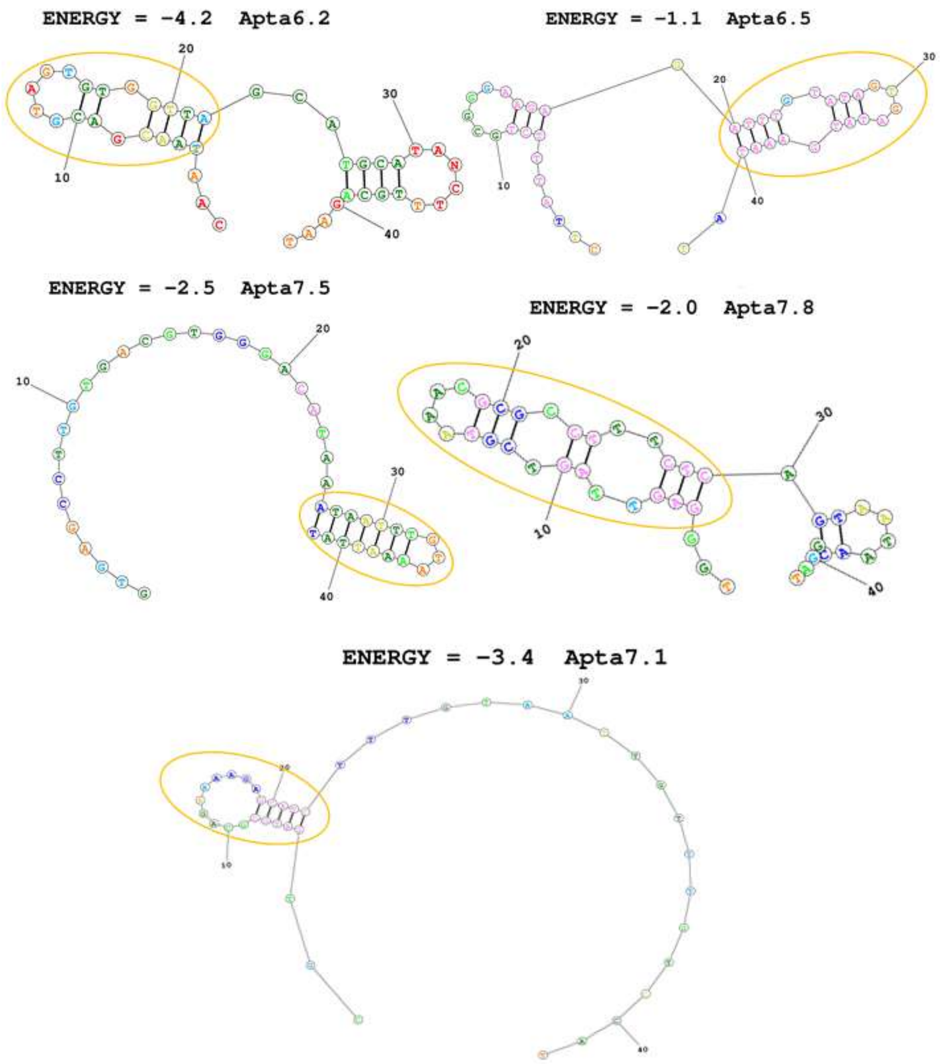

Figura 3 – Estrutura secundária predita de menor energia dos aptâmeros sequenciados e demonstrados na Tabela 2. A predição da estrutura secundária dos oligonucleotídeos selecionados foi feita no servidor RNAstructure a partir do cálculo de energia livre de ligação e do cálculo da função de partição. (https://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html).

Conclusões e perspectivas

Em linhas gerais, é possível concluir que o protocolo estabelecido para seleção de aptâmeros contra alvos proteicos imobilizados em membrana de nitrocelulose foi eficiente. Mais ajustes precisam ser feitos quanto ao número de ciclos de seleção, porém este fator varia de proteína para proteína, impossibilitando o estabelecimento de um consenso prático prévio. Para otimização da seleção, é valido também que se crie alternativas para acompanhar a afinidade de interação (Kd) do *pool* de aptâmeros selecionados em cada round pois o valor da constante permitirá acompanhar a progressão da seleção, ciclo a ciclo.

Atualmente, o pool de aptâmeros eluídos no sexto e sétimo round foram enviados para sequenciamento de nova geração na plataforma Illumina MiSeq. Estes dados irão contribuir para uma melhor caracterização e análise dos oligonucleotídeos selecionados. Após esta etapa, esperamos sintetizar estes nucleotídeos para a construção de um aptasensor que atue na detecção da neosporose bovina em diferentes fluídos corporais. Além do mais, presumimos que este mecanismo de detecção gerará um produto acessível, uma vez que os principais insumos são reagentes químicos e pequenas sondas de nucleotídeos, ambos de baixo custo. Além disso, o estabelecimento de uma plataforma para evolução molecular na seleção de oligonucleotídeos contra alvos proteicos, ainda pouco explorada em nosso território, deixará uma boa herança na busca por moléculas ativas para o desenvolvimento de novas alternativas para diagnóstico e terapia de diferentes doenças.

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CONCLUSÕES GERAIS

No presente trabalho, buscou-se criar metodologias alternativas para desenvolvimento e triagem de moléculas naturais e sintéticas para aplicação no diagnóstico de doenças bovinas. Neste sentido, três diferentes metodologias foram utilizadas com resultados satisfatórios.

Em um primeiro momento, a triagem de epítopos a partir do *phage-display* possibilitou a detecção in vitro de peptídeos antigênicos que separassem soros de animais doentes dos saudáveis. Além disso, este rastreio inicial permitiu a busca por proteínas naturais do *Trypanosoma vivax* que apresentassem sequencia antigênica similar a encontrada nos peptídeos. A metodologia forneceu uma plataforma que combina o uso de ferramentas de interação sorológica com peptídeos, através da apresentação fagos, com uma triagem computacional simples para seleção de moléculas eficientes no diagnóstico.

Em seguida, optou-se por utilizar uma abordagem completamente computacional para rastreio em larga escala de antígenos no proteoma do protozoário modelo, *Neospora caninum*. A abordagem in silico diminuiu os custos envolvidos na triagem por fago e expandiu o número de sequências proteicas avaliadas para, então, combinar as melhores regiões e construir uma proteína quimérica. Neste sentido, a eficiência desta nova molécula foi comprovada na diferenciação dos soros coletados dos animais.

Por fim, foi estabelecido uma metodologia para seleção de aptâmeros contra alvos proteicos imobilizados. Até o presente momento, tem-se comprovado a eficiência do protocolo em selecionar gradativamente, através de 7 ciclos de seleção, moléculas de DNA que interajam com a proteína de interesse. Para completa validação da metodologia, enquanto estratégia de desenvolvimento de moléculas para diagnóstico, ainda se faz necessário avaliar se as sequências de DNA selecionadas irão interagir com fragmentos da proteína-alvo em amostras biológicas coletadas dos animais infectados.

Em linhas gerais, as evidências experimentais encontradas sugerem que as três metodologias são promissoras, relevantes e eficientes no desenvolvimento de moléculas para o diagnóstico sorológico e molecular. Indo além, estas metodologias permitem a busca e o desenho de novos testes para o diagnóstico de baixo custo, otimizados para uma detecção Point-of-care (POC) para as mais diferentes doenças, sejam aqueles do setor agropecuário, ou da saúde humana.

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ANEXO I: ARTIGO DE REVISÃO PUBLICADO NA REVISTA FRONTIERS IN MICROBIOLOGY

frontiers
in Microbiology

REVIEW
published: 19 February 2021
doi: 10.3389/fmicb.2021.606360

Enlarging the Toolbox Against Antimicrobial Resistance: Aptamers and CRISPR-Cas

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In the post-genomic era, molecular treatments and diagnostics have been envisioned as powerful techniques to tackle the antimicrobial resistance (AMR) crisis. Among the molecular approaches, aptamers and CRISPR-Cas have gained support due to their practicality, sensibility, and flexibility to interact with a variety of extra- and intracellular targets. Those characteristics enabled the development of quick and onsite diagnostic tools as well as alternative treatments for pan-resistant bacterial infections. Even with such potential, more studies are necessary to pave the way for their successful use against AMR. In this review, we highlight those two robust techniques and encourage researchers to refine them toward AMR. Also, we describe how aptamers and CRISPR-Cas can work together with the current diagnostic and treatment toolbox.

Keywords: antimicrobial resistance, molecular diagnostic, alternative treatments, aptamer, CRISPR-Cas

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Edited by:
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Reviewed by:
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Yi Wang,
Auburn University, United States

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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *14 September 2020*
Accepted: *05 January 2021*
Published: *19 February 2021*

Citation:
Pereira HS, Tagliaferri TL and Mendes TAdO (2021) Enlarging the Toolbox Against Antimicrobial Resistance: Aptamers and CRISPR-Cas. Front. Microbiol. 12:606360. doi: 10.3389/fmicb.2021.606360

ANTIBIOTIC RESISTANCE CRISIS

Despite antimicrobials' impact on modern medicine since their introduction in the first part of the 19th century (Powers, 2004; CDC, 2019), resistant bacteria quickly emerged throughout the decades. Drug resistance to all available antibiotics has been detected in clinical bacteria, threatening all advances achieved within the antibiotic era and urging for alternative treatments (CDC, 2019).

Abbreviations: ALISA, Aptamer-linked immobilized sorbent assay; AMR, Antimicrobial resistance; ARG, Antimicrobial resistance genes; CRISPR-Cas, Clustered regularly interspaced short palindromic repeats, CRISPR-associated enzymes; crRNA, CRISPR RNA; COVID-19, Coronavirus disease 2019; DNA, Deoxyribonucleic acid; FDA, Food and Drug Administration; HIV, Human immunodeficiency virus; KPC, *Klebsiella pneumoniae* carbapenemase; LAMP, Loop-mediated isothermal amplification; MALDI-TOF MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MRSA, Methicillin-resistant *Staphylococcus aureus*; NDM, New Delhi metallo-β-lactamase; NGS, Next-generation sequencing; PAM, Protospacer adjacent motif; PBP, Penicillin-binding protein; RNA, Ribonucleic acid; RNP, Ribonucleoprotein; RT-qPCR, quantitative reverse transcription PCR; SARS, Severe acute respiratory syndrome; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; SELEX, Systematic evolution of ligands by exponential enrichment; sgRNA, single-guide RNA; siRNA, Small interfering RNA; SNP, Single-nucleotide polymorphism; TALEN, Transcription activator-like effector nucleases; tracrRNA, trans-activating CRISPR RNA.

Bacteria have developed resistance mechanisms to avoid, disrupt, eject, or resist the currently used antimicrobials (**Box 1**). They can be intrinsically resistant to antibiotics by using structural or functional inherent bacterial features or acquire resistance via genetic mutations or by horizontal transference of genetic elements (Blair et al., 2015; Munita and Arias, 2016).

The spread of bacterial resistance mechanisms has been much faster than the development of new treatments. New investments on antimicrobial research have been discouraged due to their elevated production costs and long-term development process (Adams and Brantner, 2010). On top of that, the misuse and over-prescription of antibiotics, which stem from uncertainties in diagnosis, contribute to the antimicrobial resistance (AMR) crisis escalation (Llor and Bjerrum, 2014; Malik and Bhattacharyya, 2019). The lack of rapid diagnostic tools directly affects initial treatment decisions, which might lead to empirical treatment guided only by clinical presentation (Fischer et al., 2004; Leekha et al., 2011).

Phenotypic-based diagnostics are currently considered as gold standards in AMR assessment. The "catch-all" resistance characteristic of phenotypic tests enables the evaluation of microbial susceptibility in a relatively unbiased way (Mitsakakis et al., 2018). Although efforts have been made to provide quick phenotypic tests (~7 h) (Pancholi et al., 2018) to better guide antibiotic treatment, the most used techniques still require microorganism culture, with a turnaround time of around 18 h. This delays the availability of the AMR profiles, which might be accessible up to 72 h after sample collection (Leekha et al., 2011). Besides time-to-result limitation, phenotypic tests generally require laboratory structure (Mitsakakis et al., 2018). Therefore, quicker and accessible diagnostic tools are imperative to guide the first medical decisions regarding antimicrobial therapy prescription worldwide.

MOLECULAR APPROACHES

The search for more precise molecular diagnostic tools with quicker turnaround times has been encouraged to better guide clinical practice and public health policies. The recent global SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) outbreak has shown that in a matter of weeks, diagnostic centers have been overloaded with patients' samples and quick result release is required for viral spread control. So far, until 21 December 2020, SARS-CoV-2 virus infected 75,704,857 individuals, with 1,690,061 deaths worldwide[1]. We dare to draw here a parallel between SARS-CoV-2 and the AMR crisis. Currently, AMR infections cause around 700,000 deaths per year (O'Neil, 2016). In both cases, an early diagnosis would give trustworthy information for discrimination and contention of the causative agent. With alarming death numbers, the exploration of alternative treatments and diagnostics comes into the spotlight as an attempt to revert the current scenario caused by AMR.

Different molecular tools have been employed as a diagnostic to identify infectious disease agents and their resistance profile. RT-qPCR (quantitative reverse transcription PCR) and NGS (Next-Generation Sequencing) have been currently playing a key role in the diagnostics of the SARS-CoV-2, different from what happened in the 2002 SARS outbreak (Sheridan, 2020). qPCR is also an outstanding tool for the molecular detection of antimicrobial resistance genes (ARGs) (Waseem et al., 2019) directly from patient samples such as urine, blood, and cerebrospinal fluid (Singh et al., 2017). In addition to qPCR, metagenomic, LAMP (Loop-mediated isothermal amplification), and whole genome sequencing approaches not only characterize pathogens at the species level but also detect ARGs (Zankari et al., 2012; Dekker, 2018; Ota et al., 2019).

The molecular diagnostic tools described above offer an abundant panel to recognize DNA and RNA of infectious microorganisms. Complementarily, proteomics- and metabolomics-based techniques have been gaining momentum into the clinical molecular diagnostic field, for instance, MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry) (Patrinos et al., 2017). However, these tools require expensive equipment and laboratory structure, hampering their wide implementation as *in loco* diagnostic tools.

Aptamers and CRISPR-Cas (clustered repetitive interspaced short palindromic repeats, CRISPR-associated enzymes) systems have been slowly gaining support in clinical diagnosis and treatment of infectious diseases. Both can be employed as an onsite diagnostic tool with a quick turnaround time, which makes them more interesting than other methods targeting proteins or nucleic acids. Therefore, reviewing these two robust techniques attempts to encourage molecular biologist researchers to develop and refine clinical molecular tools against ARGs. Those methods do not necessarily intend to substitute the already implemented diagnostic approaches, but to stimulate their combination to circumvent antibiotic misuse. Also, their application in therapy will be reviewed to help paving the way for their use as treatments against multidrug-resistant agents.

APPLICATION OF APTAMERS INTO DETECTION AND NEUTRALIZATION OF AMR FACTORS

A substantial boost in aptamer application in research and clinical institutes has been noted worldwide (McKeague et al., 2015). Also called chemical antibodies (Toh et al., 2015), most aptamers interact with their targets in a constant equilibrium with binding affinities up to 1 pM (Ha et al., 2017). They offer a cheap large-scale production with chemical modifications, low or no immunogenicity, small size (close to 3 nm), flexibility in tridimensional structure, and great stability in different conditions of pH, temperature, and organic solvents (Yoon and Rossi, 2018). Also, aptamers interact with high sensitivity toward their targets, being able to discriminate a single amino acid mutation (Chen et al., 2015). Due to their small size, aptamers reach cavities that are often not accessible to monoclonal antibodies and, therefore, penetrate cell tissues more easily (Xiang et al., 2015). In this process, intracellular

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[1] https://covid19.who.int/

BOX 1 | Antimicrobial resistance mechanism.

Resistance mechanisms date back to thousands of years and have been probably used to endure the presence of toxic compounds present in nature—including antimicrobials derived from different microorganisms, while they also provide alternative cellular functions (Allen et al., 2010; D'costa et al., 2011). Bacteria can evade antimicrobials via reduction of drug intracellular concentration either by low membrane permeability or through antibiotic efflux; target modification by genetic mutation or post-translational modification; and inactivation of the antibiotic by hydrolysis or its modification (Blair et al., 2015). With the introduction and constant presence of antimicrobials in medical care, agriculture and animal health, the spread of resistant microorganisms and the evolution of their defense strategies have been accelerated (**Figure 1**; CDC, 2019). From all resistance mechanisms, genes responsible for antibiotic inactivation and target alteration (**Figure 1** I and III, respectively) are commonly spread by plasmids and phage transduction (Munita and Arias, 2016; Calero-Caceres et al., 2019). Antibiotic inactivation is a usual strategy adopted for instance against beta-lactams and aminoglycosides. Beta-lactams can be hydrolyzed by enzymes encoded by the *bla* genes (beta-lactamase genes), such as bla_{TEM}, bla_{KPC}, and bla_{OXA}. Aminoglycosides, by its turn, are chemically inactivated by mainly three biochemical reaction, named adenylation, acetylation, and phosphorylation catalyzed by the enzymes nucleotidyltransferases (ANT), acetyltransferases (AAC), and phosphotransferases (APH), respectively (Doi et al., 2016; Munita and Arias, 2016; Bush and Bradford, 2019). An advantage of the hydrolysis over the chemical alteration strategy is the requirement of water instead of chemical compounds as a co-substrate, which ease enzyme activity outside the cell. Target alteration can be achieved by four main strategies, affecting several antimicrobials (not limited to the examples), as follows: (i) Target protection. One of the best-studied examples involves the determinants Tet(M) and Tet(O), which confers resistance to tetracycline. They interact with the ribosome and dislodge the drug from its binding site. (ii) Mutation of the antimicrobial target site. The development of mutations in the chromosomal genes *gyrA-gyrB* and *parC-parE* codifying for DNA gyrase and topoisomerase IV, respectively, promotes resistance against fluoroquinolones. (iii) Enzymatic alteration. Macrolide resistance is acquired by *erm* genes (erythromycin ribosomal methylation), which codify enzymes responsible for 50S ribosomal subunit methylation. This alteration weakens the binding of the erythromycin to the ribosome. (iv) Replacement/bypass of the target. Beta-lactam resistance is frequently acquired by Gram-positive microorganisms via *mec*A gene. The gene encodes an exogen penicillin-binding protein (PBP2a) that has low affinity for the beta-lactams, opposite to what happens to endogen PBPs (Munita and Arias, 2016).

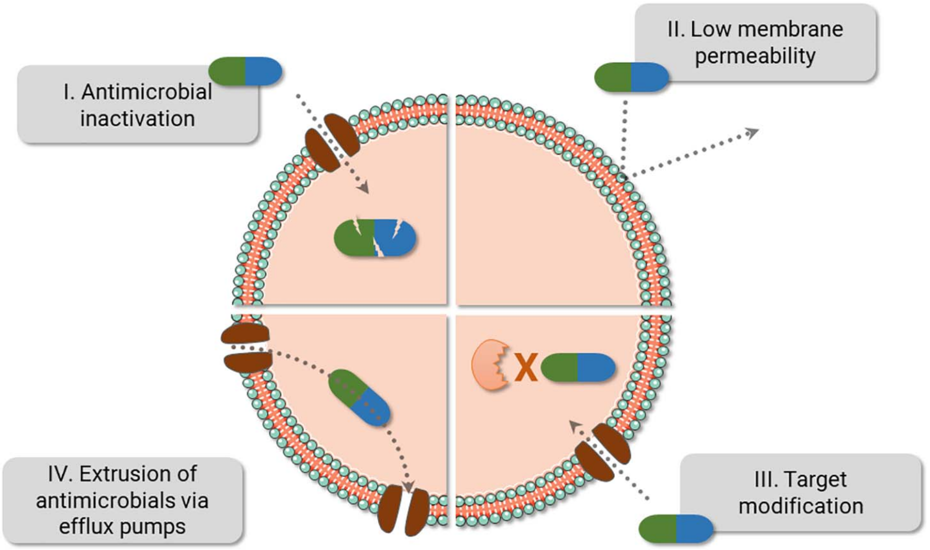

FIGURE 1 | Bacteria fight back antimicrobials. The main bacterial resistance mechanisms are displayed above. Of note, resistance against one class of antibiotics can be achieved using different strategies. For instance, resistance against beta-lactams can be caused by PBP2a (III), beta-lactamase (I), or by a combination of different mechanisms, i.e., reduction of porins (II) and the use of beta-lactamase (I). Also, efflux pumps can expel several antibiotic classes (IV) and are frequently present in multidrug resistance bacteria.

aptamers could be internalized by either a clathrin-dependent or -independent mechanism and co-localized in subcellular compartments, directly associated to its target, as reviewed in Yoon and Rossi (2018; **Figure 2**).

Single-stranded oligonucleotides can assume several secondary conformations such as hairpin, loop, pseudoknot, and G-quadruplex, which assure a unique folding for each sequence and allow interaction with specific sites (Yunn et al., 2015). DNA and RNA aptamers have similar binding characteristics, although DNA nucleotides have lower operating cost and offer greater stability than RNA, which in turn have greater versatility in their three-dimensional structures that directly affect target affinity.

Aptamer selection commonly occurs via a randomized process of systematic evolution of ligands by exponential enrichment (SELEX), firstly reported in 1990 (Ellington and Szostak, 1990; Tuerk and Gold, 1990). SELEX is based on iterative rounds of incubation of the oligonucleotide pool with the target, frequently divided into four stages: incubation, partition, recovery, and amplification. Also, aptamer–target affinity slowly increases alongside the rounds until the most specific aptamer is selected. SELEX allows greater versatility of binding conditions, which favors the adaptation of selected oligonucleotides to different cellular and non-cellular environments (McKeague et al., 2015).

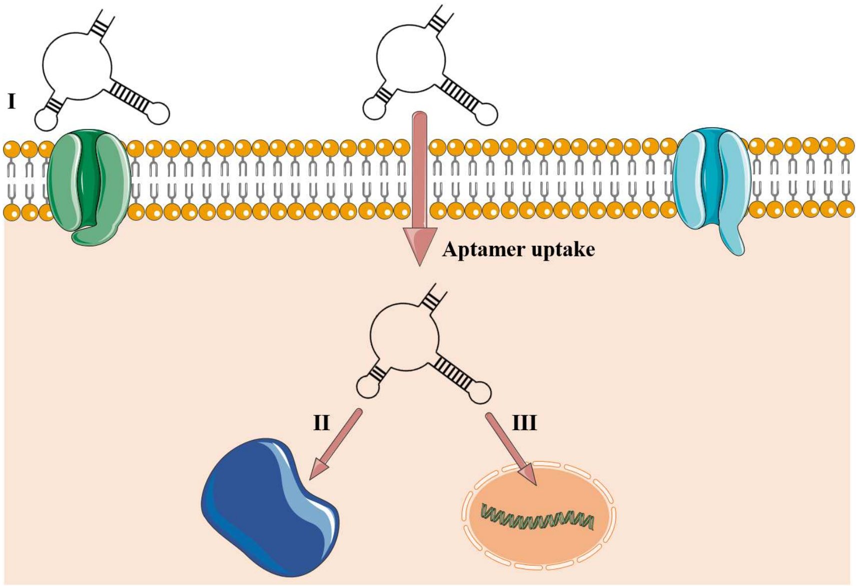

FIGURE 2 | Aptamer–target interactions in extra- and intracellular environments. (I) Aptamer recognizing cell membrane receptor or surface protein. The aptamer is uptaken into the cellular environment and can be located in some subcellular compartments such as lysosome, mitochondria, Golgi, and endoplasmic reticulum or guided to the nucleus in eukaryotic cells (Yoon and Rossi, 2018) or be stored into the cytoplasm of prokaryotic cells. (II) Cytosolic intracellular aptamers recognizing circulating proteins. (III) Nuclear intramers interacting directly with genetic elements. Aptamers' uptake is mediated to the intracellular environment by a clathrin-dependent mechanism. Clathrin is a group of proteins present in membrane cavities that assist in membrane vesicle formation during the molecule internalization by endocytosis.

Aptamer selection against many biological and chemical targets has been described (Qiao et al., 2018; Dalirirad and Steckl, 2020), along with its use in drug development (Esposito et al., 2018), bioimaging (Kim et al., 2019), food inspection (Duan et al., 2016), genetic modulation (Mol et al., 2019), and as a delivery vehicle (Zhuang et al., 2020). In antiviral therapy, G-3 aptamer dually inhibits HIV-1 cell replication both by blocking virus entrance via CCR5 receptor and by delivering a siRNA that decreases HIV-1 cytoplasmatic traffic (Zhou et al., 2015). Also, aptamers blocked quorum sensing and inhibited biofilm formation in *Pseudomonas aeruginosa* infections (Zhao et al., 2019). Even with many progresses, there is currently only one aptamer approved for clinical use, named pegaptanib. It is the main component of Macugen®, released by the FDA in 2004 to treat age-related macular degeneration (Gragoudas et al., 2004). Recently, there have been 10 therapeutic aptamers at different stages of clinical trials (Kaur et al., 2018), most of them employed to inhibit protein–protein interaction or act as antagonist (Yunn et al., 2015). To date, there is no aptamer currently approved by FDA for diagnostic purposes, even though they fit the quality standards of the diagnostic industry: affordable, sensitive, specific, user-friendly, and robust.

Different diagnostic devices based on aptamers have been proposed, including Aptamer-Linked Immobilized Sorbent Assay (ALISA), dot-blot, lateral-flow strips conjugated to nanomaterials, and the promising aptamer-based sensors (Stoltenburg et al., 2016; Shin et al., 2018; Su et al., 2018; Xiong et al., 2020). Aptasensors can be conjugated to a wide diversity of reporter molecules without modification of their activity (McKeague et al., 2015). Signal transducers commonly conjugated to aptasensors include but are not limited to colorimetric, electrochemical, and fluorescent approaches (Bai et al., 2017; Bayrac et al., 2017; Cai et al., 2019). Linking aptamer-based biosensors with nanomaterials can increase specificity and sensitivity of target binding up to 10-fold and offers a platform for rapid point-of-care diagnostic (<1 h) (Dalirirad and Steckl, 2020). Aptasensors have well-established protocols of chemical conjugation of aptamers with color or signal-transductor molecules, such as gold, silver, platinum, iron oxide nanoparticles, or carbon nanotubes and graphene oxide (Duan et al., 2016; Dehghani et al., 2018; Gao et al., 2018; Hua et al., 2018; Das et al., 2019a; Fan et al., 2020). The application of aptasensors for disease diagnosis has been tested in different samples, e.g., plasma and spiked nasal swab (Qiao et al., 2018), cultured bacteria (Maldonado et al., 2020), and urine and serum samples (Su et al., 2018). A lateral-flow paper strip conjugated with a gold nanoparticle aptamer-based sensor was developed to onsite detection of dopamine in urine samples (Dalirirad and Steckl, 2020).

When repurposed to bacteria, aptamers could recognize them by binding to antigens or cell surface receptors, or interacting with the whole cell through unknown targets (Tang et al., 2016; Song M. Y. et al., 2017; Shin et al., 2019). Although there are uncertainties concerning the mechanisms of aptamer uptake in bacteria, a report indicates that aptamers could traffic inside bacterial cells (Afrasiabi et al., 2020), similarly to what is shown in **Figure 2**. From 2016 to 2020, several papers have reported the use of aptamers applied to diagnostics of bacterial infections

(**Table 1**). Nearly all studies employed biosensors based on DNA aptamers, which indicates that for diagnostic purposes, the DNA stability overcomes the advantage of tridimensional possibilities of RNA. It is worth mentioning that only three of these studies targeted resistant bacteria or products of ARGs. First, Fan et al. (2020) made a graphene-oxide aptasensor based on peroxidase-like activity for the detection of purified PBP2a protein, encoded by the *mec*A gene. Also, Maldonado et al. (2020) employed a fast and label-free photonic pegylated aptasensor that recognizes both pure PBP2a protein and methicillin-resistant *Staphylococcus aureus* (MRSA)-infected cells in culture. Finally, Qiao et al. (2018) detected PBP2a in *S. aureus* cells collected in clinical plasma and spiked nasal swab samples infected with MRSA strains using a single bacterial lysis step. Predominantly, the diagnostic approach using aptamer has been focusing on the detection of the whole cell instead of its biological specific components, such as proteins or toxins. Therefore, due to the well-established protein-SELEX approach, there is still room for finding highly specific aptamers that bind to proteins associated with ARGs and enrich the diagnostic toolbox.

CRISPR-CAS AS A TOOL AGAINST ANTIMICROBIAL-RESISTANT PATHOGENS

CRISPR-Cas is a ribonucleoprotein (RNP) prokaryotic complex present in 50% of the bacteria and in most archaea that behaves as a prokaryotic adaptive immune system (Makarova et al., 2015). The system confers protection against mobile genetic elements, i.e., bacteriophages, plasmids, and transposons in three coordinated phases: adaptation, CRISPR RNA (crRNA) biogenesis, and interference (Hille et al., 2018). Nearly all CRISPR-Cas system counts on an ingenious mechanism to prevent self-targeting. This includes the recognition of a short sequence called protospacer adjacent motif (PAM) during adaptation and interference stages, present only in foreign nucleic acids (Mojica et al., 2009; Marraffini and Sontheimer, 2010).

Based mainly on the signature *Cas* genes, the new classification of CRISPR-Cas systems includes two different classes, six types, and 33 subtypes (Makarova et al., 2020). The most widespread class 1 CRISPR-Cas comprises types I, III, and IV. It is characterized by effector complexes with multiple Cas proteins responsible for a coordinated action from pre-crRNA processing to target cleavage. By its turn, class 2 consists of types II, V, and VI, which contains a single-protein effector module able to recognize and cleave the targeting nucleic acid (**Table 2**; Makarova et al., 2020).

Upon unraveling the CRISPR-Cas potential of gene editing in an easier, cheaper, and flexible way compared to previously established tools [for instance, TALENs (transcription activator-like effector nucleases) and Zinc-finger nucleases], the systems have been quickly repurposed to the biomedical and biotechnology fields (Chen and Gao, 2014; Liu et al., 2018; Zhang et al., 2019; Li et al., 2020). Class 2 CRISPR-Cas has been an attractive option for gene editing as a result of the effector module's simpler architecture when compared to class 1 (Makarova et al., 2020). Target specificity and cutting activity of the nucleases can be virtually programmed to any gene of interest by means of the short-length crRNA sequence. The engineering of a single-guide RNA (sgRNA) by fusing Cas9 crRNA and tracrRNA was a benchmark for gene editing (Jinek et al., 2012), but off-target effects still hold back CRISPR-Cas full potential. Several studies have tried to overcome this drawback by employing a plethora of modifications to increase the system specificity for gene editing (Kleinstiver et al., 2016; Slaymaker et al., 2016; Chen et al., 2017; Casini et al., 2018; Lee et al., 2018). Similarly, collateral effects of some Cas enzymes, i.e., the ability to indiscriminately (*trans*-) cleave ssDNA/ssRNA unleashed by site-specific DNA/RNA (*cis*-) bound by the crRNA, may also be a limitation for gene editing. However, here, this feature has been exploited for diagnostic purposes.

The collateral effect of Cas12 and Cas13 has been used as a key step to create several diagnostic platforms, such as DETECTR, HOLMES (both using Cas12), SHERLOCK, and CARMEN-Cas13 (the last two using Cas13) (Gootenberg et al., 2017; Chen et al., 2018; Li S. Y. et al., 2018; Ackerman et al., 2020). All platforms have similar diagnostic strategies, which involve the incubation of Cas enzymes (Cas12/Cas13) along with the target nucleic acid and fluorescent ssDNA/ssRNA reporters. By detecting the target nucleic acid, the Cas enzymes *trans*-cleave the quenched-fluorescent ssDNA/ssRNA reporters inserted into the platform, generating a robust fluorescent signal from around 1-h incubation (**Figure 3**) with a good correlation with PCR-based methods (Gootenberg et al., 2017; Chen et al., 2018; Gootenberg et al., 2018). In order to achieve attomolar sensitivity, the nucleic acid detection platforms were coupled to DNA amplification steps (i.e., PCR, recombinase polymerase amplification, and loop-mediated amplification) or reverse transcriptase combined with a DNA amplification step and T7 transcription for RNA targets (Gootenberg et al., 2017; Chen et al., 2018; Gootenberg et al., 2018; Li S. Y. et al., 2018; Ackerman et al., 2020; Broughton et al., 2020). To further enhance signal sensitivity, CRISPR type III effector nuclease Csm6, responsible for non-specific RNA degradation, can be combined with Cas13 activity (Gootenberg et al., 2018).

CARMEN-Cas13 and SHERLOCK have also been explored for multiplexing assays. The first one was specifically developed for this purpose and uses droplets containing either sample or detection solution, arranged pairwise. CARMEN can test more than 4500 crRNA–target pairs on a single microfluidic chip, which represents a simultaneous detection of around 170 agents (Ackerman et al., 2020). SHERLOCK, however, explored different *trans*-cleavage ssRNA preferences of Cas13 orthologs to develop a four-channel single-reaction multiplexing (Gootenberg et al., 2018). A different multiplexing strategy is employed by FLASH, a platform that uses Cas9 to enrich low-abundance targets from complex backgrounds (including clinical specimens) before NGS (**Figure 3**; Quan et al., 2019). Both CARMEN-Cas13 and FLASH offered an important multiplexing capacity but rely on robust laboratory structure, which may impar its implementation in less developed regions. SHERLOCK, however, has demonstrated its feasibility also as a paper-based test, which

TABLE 1 | Report of literature aptamers applied in the diagnostic of bacterial infections from 2016 to 2020.

Target	Oligo	Binding affinity or LOD	References
Acinetobacter baumannii isolates	DNA	7.547 ± 1.353 pM	Rasoulinejad and Gargari, 2016
Bacillus subtilis, Citrobacter freundii, Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae, and Staphylococcus epidermidis cells	DNA	9.22–38.5 nM	Song M. Y. et al., 2017
Campylobacter jejuni cells	DNA	100 CFU ml−1	Dehghani et al., 2018
Escherichia coli and *Staphylococcus aureus* pathogenic cells	DNA	100 CFU ml−1	Xu et al., 2018
Escherichia coli ATCC cells	DNA	11.97 ± 2.94 nM	Marton et al., 2016
Escherichia coli cells	DNA	3 CFU ml−1	Jin et al., 2017
Escherichia coli cells	DNA	0.66 CFU ml−1	Hua et al., 2018
Escherichia coli O157 cells	DNA	107.6 ± 67.8 pM	Amraee et al., 2017
Escherichia coli O157:H7 cells	DNA	1.46 × 103 CFU ml−1	Yu et al., 2018
Escherichia coli whole cells	RNA	2 × 104 CFU ml−1	Dua et al., 2016
Escherichia coli whole-cells	DNA	102 CFU ml−1	Wu et al., 2017
Glycolipid antigen of *Mycobacterium tuberculosis*	DNA	668 ± 159 nM	Tang et al., 2016
Gram-negative outer membrane vesicles	DNA	25 ng/ml	Shin et al., 2019
HspX protein in tuberculous meningitis	DNA	10 pg	Das et al., 2019b
Listeria monocytogenes cells	DNA	2.5 CFU ml−1	Suh et al., 2018
Methicillin-resistant *Staphylococcus aureus* strains	DNA	1.38 × 103 CFU ml−1	Qiao et al., 2018
MPT64 antigen of *Mycobacterium tuberculosis*	DNA	0.2 fg ml−1	Bai et al., 2017
MPT64 antigen of *Mycobacterium tuberculosis*	DNA	100 CFU ml−1	Li N. et al., 2018
Mycobacterium tuberculosis cells	DNA	100 CFU ml−1	Zhang et al., 2017
Mycobacterium tuberculosis H37Ra cells	DNA	5.09 ± 1.43 nM	Mozioglu et al., 2016
Mycolactone in Buruli ulcer	RNA	1.59–73.0 μM	Sakyi et al., 2016
Mycoplasma-infected cells	DNA	Not informed	Liu et al., 2019
Neisseria meningitidis serogroup B	DNA	28.3–39.1 pM	Mirzakhani et al., 2017
PBP2a detection	DNA	20 nM	Fan et al., 2020
PBP2a in nosocomial infections	DNA	29 CFU ml^{-1}	Maldonado et al., 2020
Protein A of *Staphylococcus aureus*	DNA	11.3 nM	Stoltenburg et al., 2016
Protein A of *Staphylococcus aureus*	DNA	10 CFU ml^{-1}	Reich et al., 2017
Pseudomonas aeruginosa cells	DNA	100 CFU ml^{-1}	Gao et al., 2018
Pseudomonas aeruginosa cells	DNA	60 CFU ml^{-1}	Das et al., 2019a
Salmonella enterica serovar *typhimurium* in milk samples	DNA	3.37 × 10^2 CFU ml^{-1}	Zhang et al., 2018
Salmonella enteritidis cells	DNA	0.309 μM	Bayrac and Oktem, 2017
Salmonella enteritis cells	DNA	25 CFU ml^{-1}	Chinnappan et al., 2017
Salmonella typhimurium cells	DNA	10 CFU ml^{-1}	Duan et al., 2016
Salmonella typhimurium cells	DNA	123 ± 23 nM	Lavu et al., 2016
Salmonella Typhimurium cells	DNA	1 CFU ml^{-1}	Ren et al., 2019
Salmonella Typhimurium cells	DNA	80 CFU ml^{-1}	Wang et al., 2020
Salmonella Typhimurium cells	DNA	10 CFU ml^{-1}	Appaturi et al., 2020
Shigella sonnei cells	DNA	15.89 ± 1.77 nM	Song M. S. et al., 2017
Staphylococcus aureus and *Escherichia coli* cells	DNA	10–2,000 CFU ml^{-1}	Shen et al., 2016
Staphylococcus aureus cells	DNA	16 CFU ml^{-1}	Kurt et al., 2016
Staphylococcus aureus cells	DNA	10^3 CFU ml^{-1}	Bayrac et al., 2017
Staphylococcus aureus cells	DNA	39 CFU ml^{-1}	Cai et al., 2019
Streptococcus pyogenes cells	DNA	7 nM	Hamula et al., 2016
Streptococcus pyogenes serotype M3 cell	DNA	7.47 ± 1.72 pM	Alfavian et al., 2017
Vibrio parahaemolyticus cells	DNA	2.04e^{-9} ± 0.12 M	Ahn et al., 2018
Vibrio parahaemolyticus cells	DNA	10 CFU ml^{-1}	Sun et al., 2019

Papers recovered on the PUBMED NCBI website. Binding affinity and Limit of Detection (LOD) were represented by K_d and CFU counting, respectively.

amplifies its potential to become a widely spread quick-and-cheap ($0.61 per test) diagnostic method (Gootenberg et al., 2017). The addition of an extra step called HUDSON before SHERLOCK protocol enabled the viral detection directly from bodily fluids, contributing to the creation of a field-deployable diagnostic platform (Myhrvold et al., 2018). Its efficiency to detect bacteria directly from patient samples, however, is yet to be defined. Also, SHERLOCK might benefit from Cas13 engineering

TABLE 2 | General features of CRISPR-Cas systems based on the most well-characterized subtypes.

Type	Multisubunity RNP complex	Single protein	Signature enzyme	Seed sequence	Most common substrates		Cleavage
					RNA	DNA	
I	X		Cas3	1–5 nt and 7–8 nt		X	Single-stranded DNA cleavage
II		X	Cas9	10–12 nt		X	Blunt double-stranded DNA break
III	X		Cas10	Not defined*	X	X	Specific and non-specific ssRNA cleavage. Double-stranded DNA break
IV	Defective CRISPR-Cas loci typically lacking the effector nuclease and the adaptation module						
V		X	Cas12	∼18 nt		X	Double-stranded DNA break with staggered overhangs, non-specific ssDNA break
VI		X	Cas13	Not defined#	X		Specific and non-specific ssRNA cleavage

*Seed sequence: PAM-proximal sequence in which full complementarity is required for CRISPR-Cas interference. As (i) some CRISPR-Cas subtypes have promiscuous PAM sequences, (ii) the site sequence varies among the subtypes; the PAM sequences have not been displayed in this table. Gleditzsch et al. (2019) review the PAM recognition strategies for all CRISPR-Cas types and engineering approaches to change the PAM recognition sequence. *Inconsistent conclusions regarding the seed region of CRISPR type III: absence of seed or its presence in the 5′ end (Cao et al., 2016) or 3′ end (Peng et al., 2015). #Central seed region proposed for Cas13a (Abudayyeh et al., 2016; Liu et al., 2017). Table based on Semenova et al. (2011); Chen et al. (2018), Hille et al. (2018), and Makarova et al. (2020).*

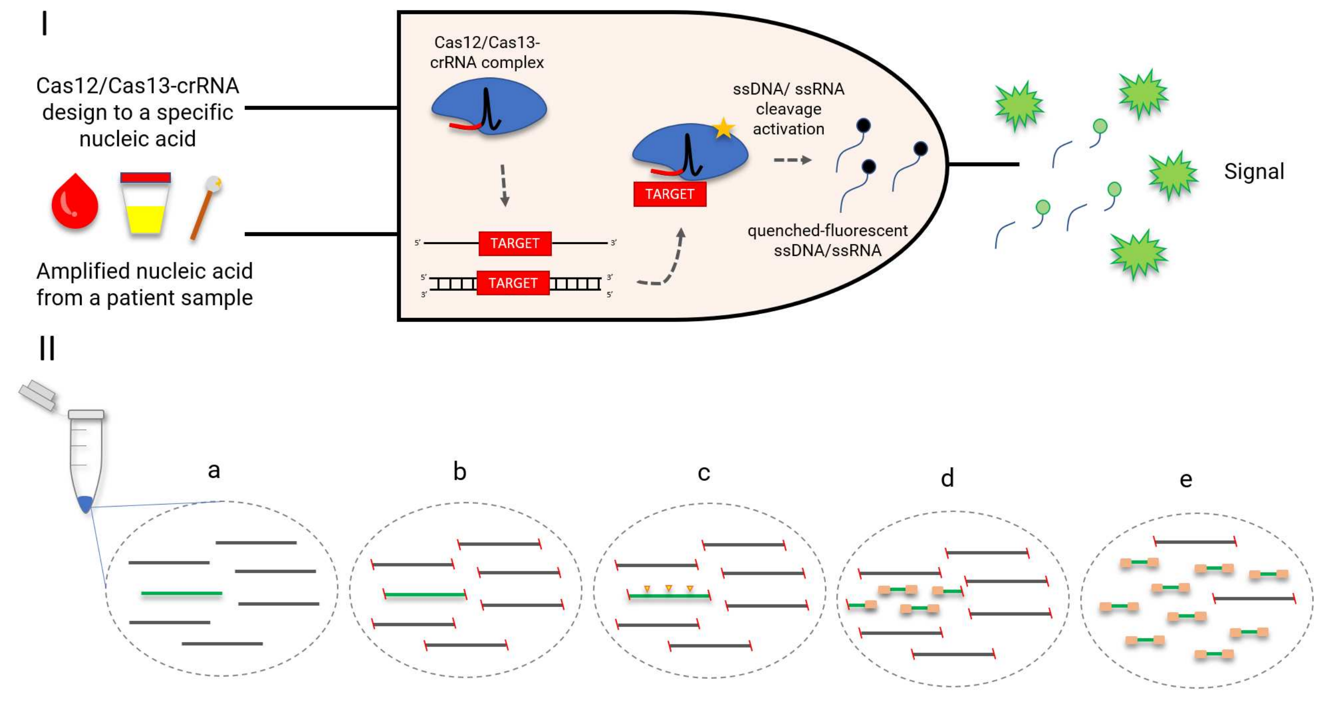

FIGURE 3 | CRISPR-Cas-based diagnostics. (I) A logic gate type "AND" of CRISPR-Cas12/13-based diagnostics. Amplified nucleic acid from patient samples can be used as input. Attomolar concentrations of RNA extracted from serum or urine samples could be detected. Without purification, Cas13 could detect RNA in samples containing up to 2% serum. The diagnostic could be performed also in cfDNA liquid biopsy samples and DNA extracted from anal swabs (Gootenberg et al., 2017; Chen et al., 2018; Gootenberg et al., 2018). A specific crRNA design to match a desired target, for instance, an antimicrobial resistance gene, is coupled with the Cas12/Cas13 as a second necessary input for the diagnostic. Once the target-positive sample is identified by the Cas12/Cas13-crRNA complex, the fluorescent output is generated. The fluorescent signal is a result of a collateral *trans*-cleavage of the quenched-fluorescent ssDNA/ssRNA by Cas12/Cas13, respectively. (II) CRISPR-Cas9 enrichment for NGS detection (Quan et al., 2019). (a) DNA or cDNA sequences containing the gene of interest (green); (b) blockage of DNA extremities; (c) CRISPR-Cas9 cleaves the target gene into fragments appropriately sized for NGS (yellow arrows); (d) ligation of universal sequencing adapters; (e) enrichment of the target sequence, which is now ready for NGS.

in order to increase target preference options and therefore the multiplexing panel.

When focusing on ARGs, *Klebsiella pneumoniae* carbapenemase (KPC) and New Delhi metallo-β-lactamase (NDM) were precisely detected and distinguished from five clinical isolates of *K. pneumoniae* (Gootenberg et al., 2017). Also, HIV drug resistance mutations from 22 patient samples could be identified (Ackerman et al., 2020). As an advantage, these platforms offer a highly specific detection of single-nucleotide polymorphisms (SNPs), which can be valuable to precisely distinguish any desired resistance gene variant (Li S. Y. et al., 2018; Myhrvold et al., 2018).

Of note, the power of the platforms to face real-world challenges has been demonstrated with the detection of SARS-CoV-2 during the COVID-19 pandemic (Ackerman et al., 2020; Broughton et al., 2020) and with the FDA emergency authorization for CRISPR SARS-CoV-2 Rapid Diagnostic using SHERLOCK platform (Guglielmi, 2020).

When employed against AMR, CRISPR-Cas9, Cas3, and Cas13 have been explored as a powerful sequence-specific antimicrobial. Cell death is an expected outcome when sgRNA is directed to genes on the chromosome or plasmids containing a toxin–antitoxin system. For vectors without toxin–antitoxin, plasmid clearance or drastic copy number reduction can be achieved when targeting plasmids up to 300 copies/cell (Bikard et al., 2014; Citorik et al., 2014; Yosef et al., 2015; Kiga et al., 2020; Tagliaferri et al., 2020). Consequently to plasmid clearance in clinical isolates, other non-targeting ARGs harbored on the target vector were also eliminated, and antibiotic reusability could be confirmed in a *Galleria mellonella* infection model (Tagliaferri et al., 2020). Several variants or sub-variants of the resistance gene can be covered with one sgRNA/crRNA directed to a conserved genetic region (Kim et al., 2016; Tagliaferri et al., 2020), while selecting a sequence from a variable region may be a strategy to achieve a narrow-spectrum effect. CRISPR-Cas-mediated interference can also be fine-tuned according to the delivery approach. CRISPR-Cas delivery can be mediated by bacteriophages, and the specificity of phage–host interactions is an advantage when the target is placed in complex environments, such as microbiota (Bikard et al., 2014; Citorik et al., 2014; Yosef et al., 2015). Alternatively, the CRISPR-Cas system can be delivered via conjugative plasmid (Citorik et al., 2014; Rodrigues et al., 2019; Ruotsalainen et al., 2019). Opposite to the phage-mediated approach, lack of specific receptors for plasmid uptake during conjugation is considered as an advantage over phage delivery, as mutations in the receptors may lead to phage resistance. On the other hand, the conjugation rate is slower when compared to transduction (Ruotsalainen et al., 2019).

DISCUSSION AND FUTURE DIRECTIONS

Even with eminent demand, little has been explored of CRISPR-Cas and aptamer potential toward treatment of bacterial infection. As for CRISPR-Cas approach, Cas9 immunogenicity must be considered (Crudele and Chamberlain, 2018), as well as the definition of the most appropriate delivery method to optimize CRISPR-Cas effect in targeting bacteria within complex microbial communities. Environmental assessments may be required to evaluate risks involved on plasmid clearance and bacterial death, which can affect the frequency of non-targeting bacterial species and non-targeting plasmids. Also, with the ordinary or induced death of the targeting bacteria, CRISPR-Cas nucleic acid will naturally be released into the environment and strategies to prevent spread and horizontal transference of CRISPR-Cas system still need to be developed.

Important limitations of *in vivo* use of aptamers stem from their susceptibility to nuclease degradation and rapid elimination due to renal filtration, but chemical adjustments to the oligonucleotide structure have contributed to decrease those shortcomings (Rohloff et al., 2014). As an FDA-approved aptamer-based therapy is already a reality, we believe that the extension of this technology to other fields, including AMR, is a matter of time.

In contrast to treatment application, CRISPR-Cas-mediated diagnostic has been recently FDA-approved for detecting SARS-CoV-2, paving the way for further applications. Its high scalability and multiplexing properties are of great value for the detection and surveillance of the wide varieties of ARGs. A limitation of this approach is the target of either DNA or RNA, which confirms the presence but not the functionality of the ARGs. Aptamers by their turn target ARG products, but in order to be used as an independent diagnostic tool, increased sensitivity to attomolar levels may be required to bacterial detection in bloodstream (Kelley, 2017). As a counterpoint, the target flexibility of CRISPR-Cas, its simplicity, and the rational design of sgRNA/crRNA can be an advantage over the more complex and randomized process of aptamer selection.

We envisioned that, in the near future, CRISPR-Cas and aptamers can be combined to treat and/or diagnose resistant bacterial infections due to their aforementioned complementary characteristics. Together, those strategies have already shown to reduce CRISPR-Cas-related off-target effects in the HEK293 cell line (Zhao et al., 2020) and to increase the delivery selectivity in liver cells (Zhuang et al., 2020), and the combination was a powerful and reliable molecular sensor able to detect nasopharyngeal carcinoma biomarkers (Li et al., 2021). Whether their combined characteristics will also be beneficial for AMR diagnostics and for treating infections caused by resistant bacteria is yet to be determined. A recent study developed a strategy to recognize surface proteins on MRSA strains by aptamer and CRISPR-Cas12a-assisted rolling circle amplification (Xu et al., 2020). Still, there is a gallery of CRISPR-Cas/aptamer combinations and target bacteria to be tested, as well as further optimizations to achieve attomolar sensitivity.

Studies employing aptamer and CRISPR-Cas for diagnostics have demonstrated their ability to provide shorter turnaround time results than the gold standard AMR phenotypic tests, which can take up to 72 h to be released. This, along with the possibility of developing paper-based diagnostics, highlights the techniques' potential to be employed as a first guidance to clinical decisions related to antimicrobial use. Altogether, the molecular approaches may offer a suitable solution to circumvent antibiotic misuse in the first antibiotic prescription, currently guided only by empirical decisions.

AUTHOR CONTRIBUTIONS

HP, TT, and TM designed and wrote the manuscript. HP and TT analyzed the data. All authors contributed to the article and approved the submitted version.

FUNDING

The group received funding from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Fundação Arthur Bernardes, and the Bill and Melinda Gates Foundation (Grant No. OPP1193112). HP was supported by CNPq Ph.D. Fellowship. TT was supported by CNPq Postdoc Fellowship—Brazil (160598/2019-2).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ANEXO II: ARTIGO DE REVISÃO A SER SUBMETIDO NA REVISTA PROCESS BIOCHEMISTRY

Emerging Potential of Synthetic Biology for Food Science

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Abstract

Since the creation of the first synthetic cell, synthetic biology researches have been an important target for studies and also of bioethical concerns due to its inherent impact in several areas such as industry, agriculture, health and the environment. Due to its multidisciplinary profile integrating approaches of molecular biology, genetics, engineering and computational modelling, synthetic biology is becoming a useful tool for overcoming challenges in food science. For instance, synthetic genetic circuits are being tested for agricultural and food safety development such as biosensors for toxins and heavy metals, nutritional improvement of foods and protection of the environment. Despite its prospects, synthetic biology promotes intense biosafety discussion among researchers who request rigorous control over developed activities and a comprehensive legislation that protects the society from the misuse of its technology. Within that perspective, this review will provide an introduction to aspects of the synthetic biology as a new and promising science applied to food production and quality control. The current development of genetic engineering technology associated to food field will also be discussed. Lastly, the discussion will focus on the legal, ethical and biosecurity aspects of the use of synthetic circuits for the progress of food science.

Keywords: CRISPR, Biobrick assembly; genetic engineering; metabolic modelling; food quality

Declarations of interest: None

1. Introduction

Modern society is marked by several challenges that threaten the quality of life or even human survival. Alongside the years, the world has been facing climate changes, scarcity of natural resources, environmental pollution, emerging or re-emerging infectious diseases, and food insecurity. Simultaneously, these worries encourage the scientific community to find alternative strategies and develop chemical and biological products to better adapt to human well-being. The food sector seeks sustainable solutions to decrease the waste in food supply and the presence of microbiological and chemical contaminants, besides enhancing the quality and shelf life of its products. By its turn, synthetic biology raises as an alternative path based on biological engineering that may support the food industry to overcome those issues [71].

Synthetic biology (here called as Synbio) is a multidisciplinary technology that integrates molecular biology, genetics, and engineering with mathematical modeling and computer science to design new unnatural systems to perform biological functions [72]. Synbio has often been confused with genetic engineering due to the overlap in some DNA manipulation tools. However, Synbio is a novel engineering method that transcends conventional genetic engineering. While the latter works on an engineered genetic material, Synbio artificially creates synthetic molecules to redesign metabolic routes, or even reprogram an entire organism to control complex biological systems modifying or enhancing an existing condition [73]. In 2010, The Venter Institute announced the first minimal synthetic bacterial cell, a landmark that introduced to laypeople an emerging field of research that would later impact several areas such as industry, agriculture, health, and environment [74].

Due to its great potential for applications in different areas, Synbio funding by government sources and private institutions has increased significantly. Between 500 million and 1 billion dollars have been invested in synthetic biology research in the US since 2005 [75]. In the European Union, from 2004 to 2013, approximately 450 million euros were invested in the synthetic-biology field and over 300 million pounds in research in the United Kingdom [75]. In China, over 200 million dollars were invested in synthetic biology research from 2011 to 2015, and increased investment of one and half billion dollars has been proposed for the periods 2018 to 2022 [76]. Some outlooks indicate that Synbio will reach a global market of US $ 38,7 billion in 2020, which will also be the bottleneck in the bioeconomy development [77, 78].

According to the FAO forecast, the population will expand by 2 billion people until 2050, claiming a 70% increase in global food supply to reach this demand [79]. Thus, new and innovative solutions are required to improve agricultural productivity and ensure food security. Herein, we discuss current applications and potential of synthetic biology approaches for improving agriculture productivity, food quality, ideally attaining a sustainable and cost-effective practice, raising the interest of several biotechnology companies in the food sector that has been conquering markets and consumers. Not surprisingly, in new fields of science, several bioethical and biosafety concerns are raised, and many discussions about the control of these new techniques and products still need to be clarified.

2. Synthetic Biology techniques applied to food science

Several techniques were developed throughout the years to enlarge the toolbox of synthetic biology approaches. Some of them are based on linkage of biological parts using restriction enzymes, assembly of several DNA fragments, genome edition by “copying” and “pasting” mechanism, or modeling metabolic routes in a living system, as we can see illustrated in Figure 1. In this section, we will describe some of the techniques applied at Synbio research with attention to food research.

2.1 Assembly method

Knight and co-workers developed the BioBricks method [80], and it works as building blocks just like Lego. In BioBrick approach, different DNA sequences are recognized as parts that are put together in a process called Standard assembly. Selected parts are ligated into a circular DNA molecule called a plasmid that acts as a vector transport system. This vector has specific sequences that flank the DNA components and facilitates its linkage to several fragments in the desired order (Figure 1a). Although BioBricks introduces itself as a quick sequence assembly technique, some drawbacks limit its range of applications. Particularly, this system is unsuitable for protein fusion due to the formation of a scar at the parts linkage which generates a stop codon interrupting the protein synthesis. On the other hand, this issue was overwhelmed by BglBrick, a new platform also capable of connecting biological parts and automating the DNA assembly by creating an innocuous nucleotide sequence when the DNA bricks are linked [81]. In 2014, Daszczuk et al. developed a fluorescent biosensor in *Bacillus subtilis*, adding to the genome of these bacteria a promoter capable of recognizing meat deterioration. A strongly activated promoter sequence, PsboA, was elucidated from the transcriptome analysis and integrated into the *B. subtilis* vector by BioBricks [82]. Boyle and co-workers constructed a synthetic system that promotes the heterologous expression of high levels of miraculin employing *Arabidopsis thaliana* as a model organism [83]. Miraculin is a protein that acts reversing the sour taste of food, sweetening its flavor at tongue by miraculin-taste receptors binding without modifying the sugar content. In this work, plant transformation vectors were developed based on the vectors of the pORE series, and linked to the synthetic region by the BioBrick technique.

Using the principles of in vitro synthetic pathway from basic building blocks-BioBricks to building modules with specific functions, You and colleagues have constructed an in vitro synthetic enzymatic pathway that can convert starch to inositol without external coenzyme supplement [84]. Inositol is important in the functional food industries, which is predominately obtained by acid hydrolysis of inositol hexakisphosphate (IP6). Also known as vitamin B8, inositol is a natural compound (widely found in dark green fruits and vegetables) indicated to reduce levels of bad cholesterol (LDL) and help control diabetes. Later, other studies have demonstrated the production of inositol also from starch [85] and other compounds, such as sucrose and glucose [86].

Kildegaard et al. [87] engineered the oleaginous yeast *Yarrowia lipolytica* for de novo production of astaxanthin by fermentation using the BioBrick technique. Astaxanthin is a red-colored carotenoid, used as food and feed additive, to improve the color of farmed fish, increase the pigmentation of egg yolks, and other feed applications. Recently, was established a novel method to produce flavor lactones from abundant non-hydroxylated fatty acids using yeast cell factories and the BioBrick technique. Oleaginous yeast *Y.*

lipolytica was engineered to perform hydroxylation of fatty acids and chain shortening via β-oxidation to preferentially twelve or ten carbons [88]. The strains could produce γ-dodecalactone from oleic acid and δ-decalactone from linoleic acid. Lactones with five(γ)- or six(δ)-membered rings and eight-to sixteen-carbons have flavors with fruity, milky, coconut, and other aromas that are widely used in the food and fragrance industries [89].

In 2016, Ma et al. obtained high expression levels of neutral protease I from *Aspergillus oryzae* by using *Pichia pastoris* as the host strain for industrial purposes [90]. This protease is widely used in the textile, food, and medical industries and which can hydrolyze proteins in soybean or other raw materials and add delicious flavor to food [91]. The recombinant expression vectors bearing two copies of the target gene were constructed using the BioBrick method. Also, Jiao and colleagues [92] developed an efficient method based on Biobrick to construct a series of vectors harboring different copy numbers of *Rhizopus oryzae* lipase (ROL) gene cassettes, which were then transformed into *P. pastoris* to generate a strain with specific copy numbers of ROL. Lipases are very attractive enzymes for use in various industrial applications, such as in the food processing industry for brewing and winemaking, dairy processing, fruit, meat, and vegetable processing [93, 94]. Moreover, this same technique was used to engineer the yeast *Saccharomyces cerevisiae* to produce resveratrol, a natural antioxidant compound used as a food supplement and cosmetic ingredient, directly from glucose or ethanol via tyrosine intermediate [95].

By its turn, Gibson Assembly facilitates the assembly of several DNA fragments in a single isothermal reaction. It was developed by Gibson [96] and promotes DNA linkage throughout a restriction enzyme mechanism without a linked scar at the assembly site. After a few experimental steps that include the generation of overlapping DNA fragments followed by nuclease digestion to create cohesive ends, the different fragments are joined and cloned into a plasmid. The efficiency in the assembly of multiple small DNA fragments into large constructs is a fundamental technology in synthetic biology [97]. Lee and colleagues used the Gibson Assembly to construct a system for expressing four different types of follistatin protein in chickens by means of the genome of *Escherichia coli* [98]. Follistatin-type protein is glycoprotein rich in cysteine residues that play a role in the prenatal and postnatal development of chickens.

The Gibson assembly method was applied to construct the heat-inducible expression plasmid in *E. coli* strain for efficient de novo biosynthesis of L-tyrosine [99]. L-tyrosine is an essential amino acid widely used in infusions, enteral and oral diets, and in health foods as a nutritional component. Foods high in dietary tyrosine include cheese, soybeans, beef, lamb, pork, fish, chicken, nuts, eggs, dairy, beans, and whole-grain [100].

The de novo biosynthesis of plant purine alkaloids, including caffeine (coffee), theophylline (antiasthma drug), theobromine (chocolate), and diverse methylxanthines using the eukaryotic microbial host *S. cerevisiae* engineered by the Gibson assembly method was presented by McKeague and collaborators [101]. These authors constructed a xanthine-to-xanthosine conversion pathway in native yeast central metabolism to increase endogenous purine flux for the production of 7-methylxanthine, a key intermediate in caffeine biosynthesis. Yeast strains were further engineered to produce caffeine through the expression of several enzymes (N-methyltransferases) from the coffee plant. These enzymes expression was responsible for redirecting the flux to an alternative pathway and developing strains that support the production of several methylxanthines [101]. The most well-known compounds of the family of methylxanthines are: caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine), and theophylline (1,3-dimethylxanthine) and are naturally present in tea leaves, yerba mate, coffee beans, cocoa beans, kola nuts and guarana berries [102]. Methylxanthines, especially caffeine, are of great interest worldwide due to their high consumption and addition in foods and beverages as functional ingredients by many manufacturers. The addition of synthetic additives to beverages has been a measure adopted for years. One example is the addition of caffeine to soft drinks, especially in cola soft drinks as a substitute for kola nut extract [102].

The use of synthetic biology has also been applied to increase β-carotene productivity in yeasts. Carotenoids, particularly β-carotene, are compounds of great interest in yeast cell factories, being widely used in the food and health industries as a feed additive and/or nutraceutical [103]. López et al. [104] constructed an integrative plasmid needed for heterologous β-carotene production in the *S. cerevisiae* strains using the Gibson assembly technology. These authors found that the conventionally built strain performed robustly, achieving higher biomass and β-carotene productivity in fed-batch cultivations when compared to *S. cerevisiae* obtained previously from adaptive evolution experiments [104].

2.2 Genome editing by CRISPR /Cas technology

Genome editing is the process in which a DNA fragment within a given system is modified by the action of enzymes known as nucleases (molecular scissors) that can replace, insert or delete specific regions of the molecule. One of the first genome-editing methods was established by Kim and colleagues in 1996 with the use of zinc-finger nucleases (ZFNs), followed by Christian et al. that used transcription activator-like effectors nuclease (TALENs), which operate under similar mechanisms [105, 106]. Another endonuclease-based system for genome manipulation recently awarded a Chemistry Nobel Prize was proposed by Jennifer Doudna and Emmanuelle Charpentier. Named Clustered Regularly Interspaced Short Palindromic Repeat-associated protein (CRISPR/Cas9) this system was first recognized as a part of the adaptive immune system of bacteria and archaea and suppressed the use of former techniques by using specific nucleases for genome modification [107, 108]. The three-component complex consists of a protein, Cas9, which acts as molecular scissors under the guidance of a small RNA called guide RNA (gRNA), which indicates the exact location of genome editing [109], as exemplified in Figure 1b. The CRISPR/Cas9 system requires no engineering steps, has multiple targeting capabilities, varying according to the gRNA, and is considered by scientists a simple, and inexpensive, and versatile technique.

In agriculture, the CRISPR system should be a promising enabler for plant genome editing. CRISPR has been used to generate crops with the desired traits, such as stress tolerance addressed by DuPont Pioneer (now Corteva Agriscience) with its engineered drought-resistant maize [110], increase in soybean productivity by changing flowering times [111], tomato editing for resistance to thermal stress and powdery mildew disease resistance [112, 113], potatoes edition for a waxy phenotype [114], fruit engineering (strawberries and apples) [115], increased grain yield in rice [116], production of mushrooms with decreased browning [117], production of rice with dense erect panicles and larger grain size [118], increased amylopectin content in maize with improved digestibility that has the potential to be commercialized [119] and modification of promoters of genes related to quantitative traits in tomatoes, such as fruit size, inflorescence branching and plant architecture [120].

Following the discovery of CRISPR, numerous multi-disciplinary studies aimed to adapt the CRISPR approach to different potential industrial applications in the agriculture, such as virus-resistant plant development [121, 122], resistance to plant diseases [123] plant nutritional improvement [114, 124], production of bioactive compounds [125-130] and physical and chemical resistance of crops [110, 131, 132] (for more information see [133]).

In addition to crops, CRISPR implementation in the animal kingdom has also occurred, such as using nCas9 to increased resistance to tuberculosis into cattle [134], editing genes in pigs to be leaner and increase pig production and save farmers money on swine going to market [135] and a knockout in chickens to remove a protein from egg whites that is known to cause some allergic reactions [136].

Li et al. [137] manipulated the γ-aminobutyric acid (GABA) shunt in tomatoes using a multiplex CRISPR /Cas9 system that targeted five key genes, resulting in GABA accumulation in both the leaves and fruits of genomically edited lines, which GABA content in the leaves 19-fold higher than that in wild-type plants. Tomato, food abundant in nutrients and metabolites, produces large amounts of GABA during fruit development [138]. This acid is considered as a health-promoting functional compound and, in plants, GABA homeostasis is important for plant growth [138]. GABA has been generally recognized as safe (GRAS) by the Food and Drug Administration. Furthermore, GABA can alleviate oxidative damage of fruits [137] and barley seedlings [139] through enhancing the activities of antioxidant enzymes. Exogenous GABA treatment significantly improves the storage performance of fruits through increasing the accumulation of citrate and amino acids [140], reducing chilling injury [141], and altering the carbon and nitrogen metabolism [142]. Therefore, GABA has great potential as an antioxidant or additive in food preservation.

In microorganisms, CRISPR has many useful advantages in the microbial industry, both natural and engineered. The CRISPR/Cas9-coupled recombineering system was also used to rapidly and efficiently engineer *the Corynebacterium glutamicum* genome. In this bacterial species, the genes *Ncgl*1221, *gabT*, and *gabP* encoding the L-glutamate transporter, GABA transaminase, and GABA permease, respectively were targeted for deletion using CRISPR/Cas system, and *gadB* was overexpressed leading to increase the production of GABA [126].

In *E. coli*, this approach has greatly facilitated genome editing for efficient de novo biosynthesis of L-tyrosine, an important amino acid widely used in food, agriculture, and pharmaceutical industries. The CRISPR-Cas9 technology was applied to eliminate L-phenylalanine and L-tryptophan pathways for their competition for the carbon flux. The *pheA/tyrR* double-gene deletion strain with the heat-inducible expression plasmid pAP-*aroG*$^{\mathrm{fbr}}$-*tyrA*$^{\mathrm{fbr}}$ was able to produce 55.54 g/L L-tyrosine by fed-batch fermentation [99]. More examples for the application of CRISPR /Cas9 in *E. coli* can be found from the efficient formation of anthocyanins as glycosylated flavonoid pigments that are of significance in the food industry, valuable as natural food and beverage colorants [143], production of β-carotene [144] and pinosylvin, a bioactive stilbene of great interest for food supplements [145].

The CRISPR /Cas9 for genome engineering and manipulation of metabolic pathways in cyanobacteria have also been reported. Li and collaborators [118] conducted knock-out of *glgc* and knock-in of *gltA/ppc* genes in *Synechococcus elongatus* PCC 7942 aimed at the production of succinic acid, an important ingredient in the food and pharmaceutical industries. These authors observed an ≈11-fold increase in production when compared with that of the wild-type cells [118].

Wu and colleagues [146] engineered the membrane of *E. coli* in both its morphological, by overexpressing membrane-binding proteins, and biosynthetic aspects, by engineering the membrane synthesis pathway by knock-in of *almgs* gene using a CRISPR /Cas9 system, as a means to study and improve its storage capacity for β-carotene, an important ingredient in the food. The combination of these two strategies had a synergistic effect, which caused a 2.9-fold increase of β-carotene specific production value (from 6.7 to 19.6 mg/g dry cell weight). This membrane engineering strategy was also applied to the β-carotene hyper producing strain CAR025, which led to a 39% increase of the already high β-carotene specific production value (from 31.8 to 44.2 mg/g dry cell weight in shake flasks) [146].

The application of the various CRISPR-Cas tools has also been used to manipulate the fermentation process along the food supply chain. The aroma profile of *S. cerevisiae* used in beer fermentation has been altered to improve taste, but with reduced alcohol percentage [147]. One study utilized wine yeasts and targeted urea production to limit the potential for carcinogen production in the fermentation process [148]. In lactic acid bacteria, that play a vital role as starter cultures in the fermentation of a variety of foods, often altering the flavor and texture in addition to aiding in preservation, the CRISPR /Cas technology was first demonstrated in 2012 by Oh and van Pijkeren. CRISPR was combined with recombineering and used as a selection marker in the probiotic *Lactobacillus reuteri*, leading to efficiencies on the order of 50–90% [149]. In this microorganism, the production of secondary metabolites with probiotic properties, such as reuterine, has been obtained through the engineering of metabolic flow pathways. Posteriorly, the technique has expanded to *Lactococcus lactis* [150, 151], *Lactobacillus plantarum* [152, 153], and *Lactobacillus brevis* [152]. This approach can also be implemented in starter culture workhorses such as *L. lactis*, *Lactobacillus bulgaricus* subsp. *delbrueckii*, and *Streptococcus thermophilus* to enhance their functional properties such as the genesis of diacetyl flavor, uptake and catabolism of amino acids, and exopolysaccharide synthesis, respectively [154]. CRISPR–Cas9 methods have also been used to remove mobile genetic elements in *L. lactis* [155] and *S. thermophilus* [156].

In a study developed by Zhou et al. [157], CRISPR/Cas9-assisted double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA) recombineering were established in *L. plantarum* WCFS1 to seamlessly edit the genome, including gene knockouts, insertions, and point mutations. These strategies were applied to engineer *L. plantarum* WCFS1 toward producing N-acetylglucosamine (GlcNAc), a precursor of glycosaminoglycan, which plays an important role in maintaining healthy cartilage and joint tissue function [158]. Thus, it is widely used as a food supplement for managing osteoarthritis [159]. Despite a few studies on lactic acid bacteria, the CRISPR /Cas system offers new possibilities for genome enhancement of probiotic bacteria and food starter cultures to improve human health and safety in the food supply chain. CRISPR-based technologies could be widely implemented to enhance the functional attributes of existing industrial strains and to develop next-generation food cultures with new properties.

CRISPR applications in other microbes such fungi have also been described. Among these studies, it is possible to mention the editing of genes in *Aspergillus*, *Myceliophthora thermophila*, and *Penicillium chrysogenum* [160-162].

CRISPR is influencing the food supply chain at every level: from starter cultures to crop and livestock improvement. In bacteria, the development of robust strains has the potential to increase the yield compared with the yield of wild-type strains. The successful applications of the CRISPR /Cas 9 system in some bacteria, plants, and animals are indicative of the potential of this genome editing tool, which may in the future revolutionize the bio-production of industrially valuable products. Furthermore, the simplicity and versatility of the CRISPR/Cas9 system have made the metabolic engineering approach simpler and more economical.

2.3 Metabolic engineering

Metabolic engineering is the manipulation of metabolic pathways to enhance the productivity of genetic and regulatory processes in a living organism. Although it is not considered a technique, the concepts complement the idea of creating synthetic organisms or systems proposed by Synbio. Metabolic engineering is widely used in the food sector for the optimization of primary and secondary metabolites, the production of microbial substances, and nutritional improvement (Figure 1c). This strategy was successfully used to produce a tomato with a high level of carotenoid by the introduction of two algal genes that efficiently directed carbon flux into astaxanthin [163] and sweet potato with a high content of that same compound by cloning of most of the carotenoid biosynthetic genes, as well as the Orange gene involved in carotenoid accumulation [164].

Due to their genetic tractability and mature high-density fermentation technologies, bacteria *E. coli* and yeast *S. cerevisiae* are the most used model organisms for metabolic engineering. Metabolic engineering was used in *E. coli* to produce L-malate [165], a four-carbon dicarboxylic acid that is used as an additive in the food, and other amino acids that are naturally produced from *Corynebacterium glutamicum*. This strategy was also used in this microorganism for the production of fruit-like ester 2-phenyl ethyl acetate, a compound with high-value flavor and applications in food industries, from glucose [166] and 2’-fucosyllactose, is one of the most abundant oligosaccharides in human milk [167].

The biosynthesis of inositol, a water-soluble vitamin B group compound, from glucose in recombinant *E. coli*, was optimized by metabolic engineering strategies [168]. Inositol has attracted the attention of the food and feed industries, where it is mainly used as an additive in drinks, in milk powder as a nutritional supplement, and in animal feed to prevent diseases [169]. Another vitamin B group, vitamin B12 extensively used in food, was biosynthesized using engineered *E. coli* strains [170]. According to the researchers responsible for the work, the metabolic modifications and optimization of fermentation conditions presented in the paper offer a basis for further improving vitamin B12 production in *E. coli* and accelerate its industrial application [170].

Natural products are widely employed in our daily lives as food additives, pharmaceuticals, nutraceuticals, and cosmetic ingredients, among others. Metabolically engineered *E. coli* has been widely used for the synthesis of these products because of many advantages including the availability of well-established tools and strategies for metabolic engineering and high cell density culture, in addition to its high growth rate [171]. Systems metabolic engineering allows the development of engineered *E. coli* strains that are capable of more efficiently producing diverse natural products, such as lycopene, a carotenoid nutraceutical possessing antioxidant and anti-cancer properties, produced by direct extraction from tomatoes, chemical synthesis, and microbial fermentation [172], β-Carotene [173], vanillin [174], astaxanthin, a reddish keto-carotenoid utilized in animal feed and food pigment [175], methyl anthranilate, a grape flavoring agent [176], heme, a porphyrin derivative essential in food supplement [177], and many other examples (for more information see [171]). Metabolic engineering has also been applied in *E. coli* for the production of steviol glycosides, a compound that has attracted the attention of the food and beverage industry due to the properties of containing no calories and taste 200–300-fold sweeter than sucrose [178].

Anthocyanins, a polyphenolic pigments which provide pink to blue colors in fruits and flowers, is used as food pigment in industries and as health-promoting substances. *S. cerevisiae* was engineered to produce anthocyanin pelargonidin 3-O-glucoside starting from glucose. Specific anthocyanin biosynthetic genes from *A. thaliana* and *Gerbera hybrida* were introduced in an *S. cerevisiae* strain producing naringenin, the flavonoid precursor of anthocyanins [179]. Moreover, *S. cerevisiae* was also engineered to produce phenolic compounds (cinnamaldehyde, cinnamyl alcohol, and hydrocinnamyl alcohol) with applications in the flavor, food, and cosmetic industry [180], fructooligosaccharides, an oligosaccharide that exhibits prebiotic properties and they are gaining attention as an important functional food ingredient [181], flavonoids (quercetin, liquiritigenin, naringenin, resokaempferol, kaempferol, and fisetin) from glucose [182], carotenoids [183, 184], astaxanthin [185], and farnesene, used as an additive in food [186]. The company Amyris developed a high-level farnesene platform in *S. cerevisiae*, rewiring the yeast central metabolism by introducing four non-native enzymes [186].

Other microorganisms have also been used in metabolic engineering for the production of compounds, such as *Y. lipolytica* used from the production of erythritol, a four-carbon sugar alcohol with sweetening properties that are used by the agro-food industry as a food additive [187], fructooligosaccharides [188], lycopene [189], polyunsaturated fatty acids, that are known an important nutraceutical for the functional food industry and linolenic acid [124]. Metabolic engineering has also been applied for the production of lycopene by *P. pastoris* X-33 [190] and production of 5-

Ketofructose, a promising non-nutritive natural sweetener, by *Gluconobacter oxydans* [191].

Natural monocyclic aromatics, which are used as flavoring agents, can be obtained through metabolic engineering of microorganisms. Vanillin, the most important flavoring agent used worldwide is produced by biotransformation of ferulic acid using *E. coli*, *Pseudomonas fluorescens* or *Streptomyces sannanensis* as production hosts [192]. Vanillin production, currently one of the most widely used flavoring compounds on the market, from cheap glucose could be successfully established in *Schizosaccharomyces pombe* and *S. cerevisiae [193].* The biosynthetic pathway leading to raspberry ketone, a primary aroma compound in raspberries, was reconstructed in *S. cerevisiae* [194]. γ-Decalactone (4-decanolide), the main volatile compound found in peaches and used as a flavoring agent, for example, in beverages, can be produced by metabolic engineering using yeasts such as *Y. lipolytica*, *S. cerevisiae*, or *Pichia etchelsii* [195].

Lactic acid bacteria, important in the food industry due to their use as starter cultures in the fermentation of a variety of foods, often altering the flavor and texture, in addition to aiding in preservation, have become targets for metabolic engineering to improve relevant phenotypes. Among the metabolic activity of the latic acid bacteria strains modulated by metabolic engineering strategies for the production of industrially valuable metabolites include: acetaldehyde, an industrially valuable metabolite, widely used for imparting aroma and flavour in dairy products especially in acetoin [196], diacetyl, which provides the typical butter aroma to the dairy products [197], exopolysaccharides, that contribute to taste, texture, mouth-feel and stability of the dairy products [198], acid lactic [199], mannitol, an important sugar alcohol with a multitude of applications in various industrial bioprocesses such as artificial sweetener,flavour modifier, anti-caking and texturizing agent [200, 201], sorbitol [202, 203], succinic acid [204], vanillin [205]. Co-production of nisin, an antimicrobial peptide, and GABA, an antioxidant agent was obtained by engineered *L. lactis* for potential application in food preservation. Liu et al. [206] metabolically engineered a nisin producing strain *L. lactis* F44 for GABA production by expression of glutamate decarboxylase and glutamate/GABA antiporter.

The interdisciplinary field of metabolic engineering is rapidly evolving with the advent of advanced systems biology, synthetic biology, and evolutionary engineering approaches. However, in the future, to further widen its scope and applications, more robust genome manipulation tools are needed for cost-effective and large scale production of metabolites of interest to the food industry.

3. Challenges, biosafety and biosecurity

The application of synthetic biology and its development in the food industry raises as a promising solution to several food problems. Currently, there are more than 50 companies on the market, delivering innovative synthetic products in the most varied branches of the food sector (Table 1). From a global perspective, these companies are unable to supply the world's market due to their small quantity and density of companies located in the western region of the United States. In general, these corporations could be grouped into five assortments of activities: agriculture and crop; alternative meat; food security; supplements and customized products; and alternative food and flavor. It is possible to note a vaster concentration of companies operating in the agricultural sector, seeking to develop more resistant and sustainable species, besides the production of alternative foods and flavorings. To date, fewer products or services operate in food security have been delivered to consumers, leaving an open niche for future companies.

Regarding food security awaken some fundamental concerns. There are both concepts interconnected into synthetic biology preliminary endeavors: biosafety and biosecurity. In general speaking, biosafety is associated with the unexpected or accidental release involved in the manipulation of modified organisms. By its turn, biosecurity could be connected with the intentional or deliberate release of synthetic microorganisms in the environment with criminal or lethal intentions, producing biological weapons [72].

Discussions dealing with the advances of synthetic biology go far beyond scientific issues since it directly impacts economic and social concerns. From an economic perspective, biotech companies endeavor to deliver innovative and unexplored synthetic products to delight a consumer fastidious demand. From a social point of view, there are discussions that towards to availability of modified products, also dealing with food safety and how synthetic resources could supply food demand according to populational growth [207, 208]. Therefore, for the improvement of synthetic biology to benefit each involved part, discussions throughout the release of synthetic organisms at environmental sources, elaboration of training programs, and rigorous standardization of protocols are mandatory [209], as well as international cooperation and treaties, among interested institutions [73]. Regulatory options should always support innovation and the development of new products, protecting the public from potential damage [210].

The use of Synbio to create new products to market raises health and environmental concerns that must be addressed with specific laws and regulations. Guidelines to regulate Synbio are complex and still require elucidations. International agreements are difficult to achieve because each government differs from its regulatory approach that is vague and criticized for not being as comprehensive [77]. The fast development and how synthetic biology have been developed are disfavoring the monitoring and creation of regulative laws that correctly control its use. Unfortunately, this fact allows many illicit activities such as biopiracy that uses genetic resources in an illegal manner and bioterrorism by creating lines of pathogenic synthetic microorganisms that can also produce lethal substances or toxins. There is also the appearance of ethical criticism by various sectors of society [77, 211].

Attempts at laws and protocols have created international agreements, including the Convention on Biological Diversity (CBD) Cartagena Protocol on Biosafety, “Nagoya Protocol”, and The World Health Organizations “Biosafety Standards Manual”. Different laws compose the U.S. regulatory system which, by its turn, regulates according to product specifications, such as its purpose and properties. There are three principal federal agencies responsible for regulating products developed with genetic engineering: the Department of Agriculture’s Animal and Plant Health Inspection Service (APHIS), the Environmental Protection Agency (EPA), which regulates crops of genetically modified plants, and pesticides and also microorganisms that produce chemicals and the Food and Drug Administration (FDA) that regulates the food, feed additives, human and animal drugs sector. If the purpose was to decrease the human disease burden, then it is likely to be regulated by the FDA under the Federal Food, Drug, and Cosmetic Act (FDCA). If it is a plant or animal disease, then it may be regulated by the US Department of Agriculture (USDA) under its plant and animal protection rules. In some cases, genetically engineered organisms will be controlled by multiple agencies within a collaborative work of these three primary agencies by testing, approving, and oversight. Outside of the US, many countries have a “process-based” regulatory system whereby genetically engineered organisms are regulated under laws specifically designed to rule derived products using biotechnology. Probably, the regulatory use of genetically engineered organisms in the United States and other countries has some comparable requirements, such as information on the organism’s molecular biology and resulting phenotype, quality control assurance, stability and safety, along with assays for detecting and monitoring the release of organisms (Warner et al. 2019).

In the European Union, there are directives (2001/18/EC and 2009/41/EU) and a regulation ((EU) 428/2009) that present guidelines for Synbio that ensure certain biosafety [212]. On the other hand, emerging countries like Brazil and India are still using synthetic biology in research without a clear discussion about this technology, letting behind any biosafety regard of genetically modified organisms used in food.

Research and industry communities are urging for specific legislation regarding synthetic biology prospects, besides an extensive social dialogue. Therefore, many policies still do not encompass synthetic biology as a field with biosafety demands. So, finding an alternative to control, to use responsible techniques and to rule out irational use should be the task of competent Organs at technical, operational, and legislative levels.

Acknowledgements The support by XXX is gratefully acknowledged.

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FIGURE LEGEND

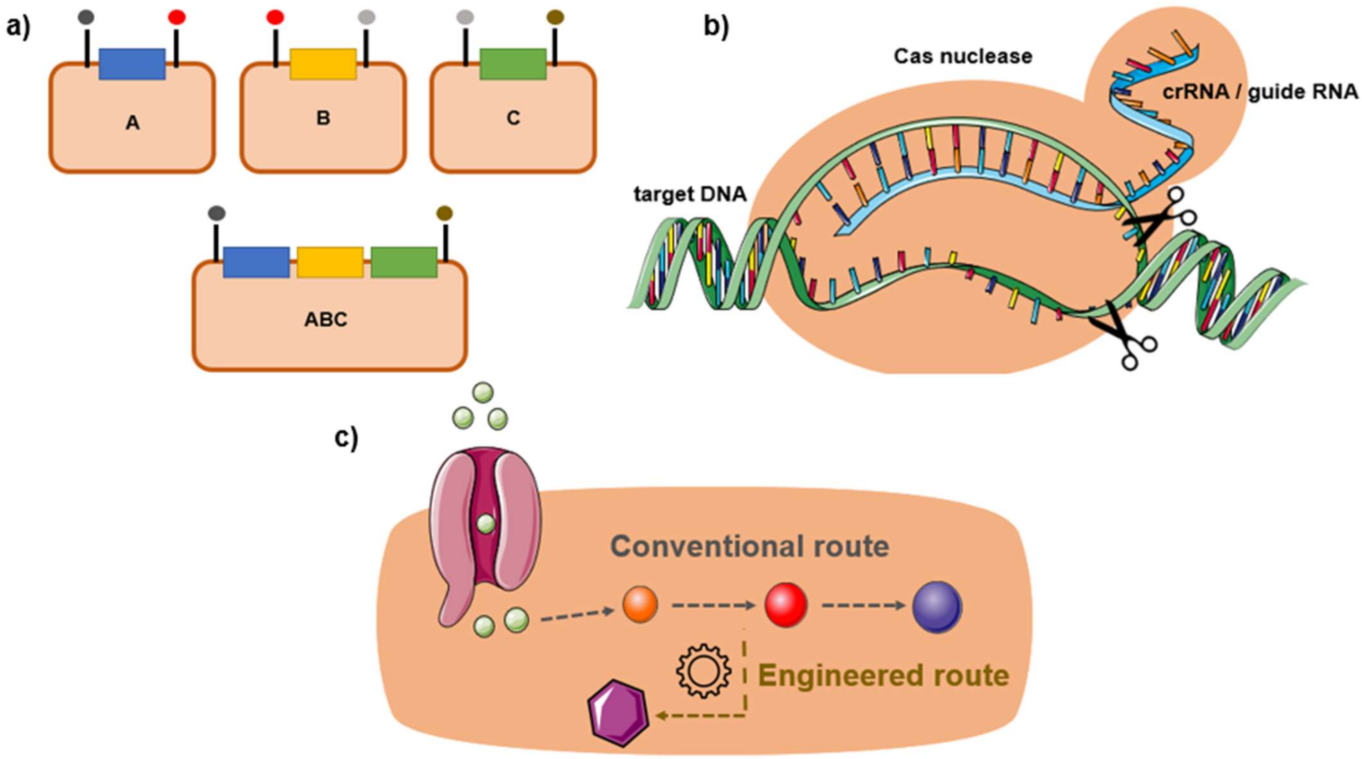
Figure 1 A general illustrated mechanism wherein the functionality of foremost practiced synthetic biology procedures at a laboratory order. a) A BioBrick assembly of synthetic parts from A, B, and C vectors connected by cohesive ends of restriction sites, here represented by color balls. A synthetic ABC vector was built with blue, yellow, and green squares ordered. b) Cas nuclease acting as a molecular scissor in a double-strand DNA fragment. crRNA (or CRISPR RNA) or guide RNA (gRNA) directs the molecular scissor to its specific sites of cut and allows the insertion of a modified fragment by a homologous and non-homologous recombinant mechanism. c) A living cell was modified to produce the desired side-product through an engineered route that starts from its conventional metabolic pathway.

TABLE

Table 1 – This table presents a summary of synthetic biology companies that are already in the food industry operating at specific niches of application. Detailed information from each company were recovered at https://golden.com/list-of-synthetic-biology-companies/, acessed at 10/21/2020.

Synbio applications at food industry	Companies
Agriculture and crop	AgBiome, agriMetis, Amyris, Benson Hill, Blue Nalu, Calyxt, Cargill, Caribou Bioscience, Concentric, Finless Food, Geltor, Modern Meadow, New Harvest, New Wave Foods, Provivi, Sound Agricultural, Syngenta, Wild Earth, Yield10 Bioscience, Zymergen
Alternative meat	Balletic Foods, Because Animals, CysBio, Future Meat, HigherSteaks, KnipBio, Meatable, Memphis Meats, Mission Barns, Mosa Meat, Shiok Meats, Super Meat, Wild Type
Food security	Calysta, Evolva
Supplements and customized products	Algama, Arzeda, Clara Foods, Evolva, Geltor, Global Bioenergy, Joywell, Motif Foodworks, Proterro, Tate & Lyle, Triton, Wild Earth
Alternative food and flavors	Algama, Amai Proteins, Amyris, Arzeda, Because Animals, Burcon NutraScience,Clara Foods, CysBio, DSM, Finless Food, Impossible Foods, Just, New Culture, Novozymes, Perfect Day Foods, Shiok Meats, Triton

FIGURE 1


a)
A
B
C
ABC
b)
Cas nuclease
crRNA / guide RNA
target DNA
c)
Conventional route
Engineered route