UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
FACULDADE DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

Diagnóstico, tipagem molecular e avaliação do perfil de resistência de cepas *Treponema pallidum*

JÚLIO HENRIQUE FERREIRA DE SÁ QUEIROZ

Dourados - MS
2024

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Área do CNPq: 40101096

Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Faculdade de Ciências da Saúde da Universidade Federal da Grande Dourados (UFGD), para obtenção do título de Doutor em Ciências da Saúde

Área de concentração: Doenças Crônicas e Infecto-Parasitárias

Orientadora: Profª. Drª. Simone Simionatto

Dourados - MS
2024

Dados Internacionais de Catalogação na Publicação (CIP).

Q3d Queiroz, Júlio Henrique Ferreira De Sá

Diagnóstico, tipagem molecular e avaliação do perfil de resistência de cepas Treponema pallidum [recurso eletrônico] / Júlio Henrique Ferreira De Sá Queiroz. -- 2024.

Arquivo em formato pdf.

Orientadora: Simone Simionatto.

Tese (Doutorado em Ciências da Saúde)-Universidade Federal da Grande Dourados, 2024.

Disponível no Repositório Institucional da UFGD em: https://portal.ufgd.edu.br/setor/biblioteca/repositorio

1. Sífilis. 2. Caracterização molecular. 3. Resistência aos antimicrobianos. 4. DNA treponêmico. 5. Técnicas moleculares. I. Simionatto, Simone. II. Título.

Ficha catalográfica elaborada automaticamente de acordo com os dados fornecidos pelo(a) autor(a).

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AGRADECIMENTOS

A construção do conhecimento científico é acompanhada por momentos e pessoas especiais, e cada uma delas, abaixo, fez parte e me ajudaram nessa minha jornada científica.

À minha família, aos meus pais (Lenir e José Jaime), aos meus irmãos (Pamela e Jaime), minha esposa (Ludmila), o Simba e o Paçoca por sempre me apoiar. A família é tudo!

Aos meus amigos e familiares que foram muito importantes durante o desenvolvimento da tese.

À minha orientadora, Profa. Dra. Simone Simionatto, por toda orientação e condução nessa minha jornada científica, como também por acreditar no meu potencial científico.

Meus agradecimentos aos integrantes do Grupo de Pesquisa em Biologia Molecular de Microrganismos (GPBMM) – Tiago, Michele, Gleyce, Márcia, Kesia, Érica, Anny, Julia, Marcelo, Gabriel, Gerlaine, Taynara, Laís, Bruno, Emily, Vinicius, Mayara, Junio, Andressa e Natalia. Muito obrigado por todo auxílio e apoio tanto no laboratório quanto nas reuniões do grupo. As enfermeiras Regina, Ilma, Carol Rangel e Karla pelo auxílio nas coletas das amostras clínicas. Os acadêmicos Emily, Eduardo e Camila pela colaboração no processamento das amostras clínicas dos postos de Saúde e Hospital Universitário de Dourados.

Muito obrigado ao SAE (Serviço Ambulatorial Especializado) e CTA (Centro de Testagem e Aconselhamento) de Dourados-MS por todo apoio na abordagem dos pacientes. As enfermeiras Débora, Patrícia e Stephanie. Um agradecimento especial à farmacêutica Glaucia, à coordenadora Aurenita Barbosa e às médicas Dra. Cindi e Dra. Luiza pelo auxílio nas avaliações dos pacientes e nas coletas das amostras clínicas. Agradeço aos amigos do grupo de pesquisa, às enfermeiras Anny, Andrea e Érica, pelo auxílio nas coletas das amostras, e aos acadêmicos Gabriel, Taynara, Tiago, Lucas e Bruno pela colaboração no processamento das amostras clínicas.

Ao Grupo de Pesquisa de Tuberculose coordenado pelo Prof. Dr. Júlio Croda por todo aprendizado durante os 10 meses na contribuição do projeto: “Estratégias para controle da tuberculose nas prisões”, e aos integrantes da equipe da TB: Paulo, Bruna, Flora, Mariana,

Raphaele, Camila, Roberto, Andrea, Alessandra, Alan, Flávia e Neia. Muito obrigado por todo aprendizado e conhecimento!

As técnicas do Laboratório em Pesquisa em Ciências da Saúde (LPCS) da UFGD, Flora, Mariana, Anália e Cleide; e a equipe do COVID-19 do LPCS, Lujan, Renata, Cristiane, pelos conselhos e café da manhã caprichado. Aos professores do LPCS, Prof. Dr. Fábio Negrão, Profa. Dra. Herinthan, Profa. Dra. Silvana e Profa. Dra. Kely Brabes, pelos ensinamentos passados durante os encontros no LPCS.

Aos secretários da pós-graduação em Ciências da Saúde da UFGD, a Geni e o Anderson, por todo auxílio e conselhos.

À Universidade Federal da Grande Dourados e ao Programa de Pós-graduação em Ciências da Saúde da UFGD por todo apoio e estrutura oferecida para a execução da minha tese.

Agradecimento à Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado do Mato Grosso do Sul (Fundect) (Processo número 71/700.102/2020) pela bolsa de doutorado concedida durante os 3 anos.

EPÍGRAFE

"Só existem dois dias do ano em que
nada pode ser feito: um se chama
ontem; o outro, amanhã.
Portanto hoje é o dia certo para amar,
acreditar, fazer e, principalmente, viver."

(DALAI LAMA)

LISTA DE ABREVIATURAS E SÍMBOLOS

BIGSdb	*Bacterial Isolate Genome Sequence*
CDC	*Centers for Disease Control and Prevention*
CEP	Comitê de Ética em Pesquisa
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
DNA	Ácido Desoxirribonucleico
ECDC	*Enhanced Centers for Disease Control and Prevention*
EDTA	Ácido Etileno-Diamino-Tetracético
FUNDECT	Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul
MS	Mato Grosso do Sul
MLST	Tipagem da Sequência Multilocus
PBS	Tampão Fosfato-Salino
PCR	*Polimerase Chain Reaction*
qPCR	*Quantitative Polimerase Chain Reaction*
RNA	Ácido Ribonucléico
RFLP	Polimorfismo do Comprimento do Fragmento de Restrição
SUS	Sistema Único de Saúde
VDRL	*Venereal Disease Research Laboratory*

Diagnóstico, tipagem molecular e avaliação do perfil de resistência de cepas *Treponema pallidum*

RESUMO

A sífilis é uma infecção sexualmente transmissível curável que teve um aumento expressivo de casos no Brasil nos últimos anos, o que representa um problema de saúde pública persistente. Desta forma, implementar ações que visem um melhor acesso ao diagnóstico, tratamento e monitoramento na atenção primária à saúde são fundamentais para o controle desta doença infecciosa. Por isso, o desenvolvimento das técnicas moleculares para a identificação de *Treponema pallidum* subespécie *pallidum*, o agente causador da sífilis, contribui para uma melhoria no diagnóstico. Além disso, a caraterização molecular é uma alternativa para o conhecimento e compreensão da transmissão dos treponemas no Brasil. Essa pesquisa teve como objetivo o desenvolvimento de métodos para o diagnóstico molecular da sífilis através da Reação em Cadeia da Polimerase (PCR) e da PCR quantitativa (qPCR). Das amostras clínicas positivas na PCR convencional e na qPCR, foram submetidas à caracterização molecular de *T. pallidum* pelo método da tipagem *Enhanced Centers for Disease Control and Prevention* (ECDC) e à avaliação do perfil de resistência aos macrolídeos e às tetraciclinas. Durante junho/2018 a dezembro 2022, 23 participantes com suspeita de lesões sifilíticas primária e secundária aceitaram participar do estudo, e 18 participantes tiveram as amostras clínicas das lesões e sangue total coletados. Nas duas amostras clínicas (lesão e sangue) desses 18 participantes, foram realizadas as técnicas moleculares PCR convencional e qPCR para detecção do DNA treponêmico. Das 36 amostras clínicas testadas, 16 (oito de lesões e oito de sangue) tiveram o DNA de *T. pallidum* detectado pela qPCR. Por outro lado, a PCR convencional identificou o DNA treponêmico em 11 amostras clínicas, oito de lesões e três de sangue. Os quatro subtipos de *T. pallidum* identificados pelo método ECDC foram: 14d/g, 14d/c, 15d/c, e 15d/e. O perfil de resistência à azitromicina foi identificado em 3/5 (60%) dos participantes com sífilis primária positivos na qPCR. Dessas amostras, duas (66,6%) continham a mutação A2058G e uma amostra não tinha a mutação. Nenhuma mutação A2059G foi encontrada. Há poucos estudos de epidemiologia molecular de *T. pallidum*, e ainda mais escassos nos países com taxas elevadas de casos de sífilis, como os países americanos. O que reforça a importância de mais estudos para a identificação do perfil molecular e de resistências em *T. pallidum*.

Palavras-chave: Sífilis. Caracterização molecular. Resistência aos antimicrobianos. DNA treponêmico. Técnicas moleculares.

Diagnosis, molecular typing and evaluation of the resistance profile of *Treponema pallidum* strains

ABSTRACT

Syphilis is a curable sexually transmitted infection that has seen a significant increase in cases in Brazil in recent years, representing a persistent public health problem. Therefore, implementing actions aimed at better access to diagnosis, treatment, and monitoring in primary health care is fundamental to controlling this infectious disease. The development of molecular techniques for the identification of *Treponema pallidum* subspecies *pallidum*, the causative agent of syphilis, contributes to an improvement in diagnosis. Furthermore, molecular characterization is an alternative to understanding the transmission of treponemas in Brazil. This research aimed to develop methods for the molecular diagnosis of syphilis through Polymerase Chain Reaction (PCR) and quantitative PCR (qPCR). The positive clinical samples in conventional PCR and qPCR were subjected to molecular characterization of *T. pallidum* using the Enhanced Centers for Disease Control and Prevention (ECDC) typing method and evaluation of the resistance profile to macrolides and tetracyclines. From June 2018 to December 2022, 23 participants with suspected primary and secondary syphilitic lesions agreed to participate in the study, and 18 participants had clinical samples of the lesions and whole blood collected. Conventional PCR and qPCR molecular techniques were performed. The two clinical samples (lesion and blood) from these 18 participants were tested to detect treponemal DNA. Out of the 36 clinical samples tested, 16 (eight from lesions and eight from blood) had *T. pallidum* DNA detected by qPCR. Conversely, conventional PCR identified treponemal DNA in 11 clinical samples, eight from lesions and three from blood. The four subtypes of *T. pallidum* identified by the ECDC method were: 14d/g, 14d/c, 15d/c, and 15d/e. The azithromycin resistance profile was identified in 3 out of 5 (60%) of participants with primary syphilis positive in qPCR. Among these samples, two (66.6%) contained the A2058G mutation, and one sample did not have the mutation. No A2059G mutation was found. There are few studies on the molecular epidemiology of *T. pallidum*, and even fewer in countries with high rates of syphilis cases, such as the American countries. This reinforces the importance of further studies to identify the molecular profile and resistance in *T. pallidum*.

Keywords: Syphilis. Molecular characterization. Antimicrobial resistance. treponemal DNA. Molecular assays.

SUMÁRIO

1 INTRODUÇÃO

A incidência da sífilis aumenta em todo mundo, com uma estimativa de 7,1 milhões de novos casos em 2020 (OMS, 2021), o que têm elevado o nível de preocupação sobre essa infecção sexualmente transmissível. No Brasil, os casos de sífilis adquirida, em gestantes e congênita vem aumentando, com a taxa de detecção passando de 14,1 para 99,2 (por 100 mil habitantes) para sífilis adquirida, de 5,7 a 32,4 (por 1000 nascidos vivos) para a sífilis em gestantes, e de 4 para 10,3 (por 1000 nascidos vivos) para sífilis congênita durante os anos de 2012 a 2022 (Brasil, 2023).

O diagnóstico da sífilis é realizado através dos testes sorológicos, manifestações clínicas e histórico do paciente. No entanto, os testes sorológicos apresentam algumas limitações, como uma baixa sensibilidade no início da infecção e a falta de capacidade de diferenciar os casos de sífilis tratada da infecção ativa (Peeling *et al.*, 2023). Essas limitações reforçam a importância do desenvolvimento de novos métodos de diagnóstico, cuja grande promessa são os testes moleculares.

O cultivo difícil e oneroso *in vivo* (em coelhos) e *in vitro* (células epiteliais de coelhos) dificulta o estudo de *T. palldium* (Edmondson; Hu; Norris, 2018). Portanto, a tipagem molecular tem se mostrado um método eficaz e pode contribuir na epidemiologia das infecções humanas. Por isso, o desenvolvimento de métodos moleculares para a diferenciação das cepas de treponemas fornecerá importantes informações sobre a transmissão destas cepas através das populações, contribuindo para uma melhor compreensão da aquisição e transmissão da doença.

O primeiro método da tipagem molecular de *T. pallidum* foi desenvolvida por Pillay *et al.* (1998) e baseou na variabilidade do gene da proteína ácida de repetição (arp) e dos genes *tpr* (*T. pallidum repeat*) da subfamília II *(*tprE *[tp0313],* tprG *[tp0317]* e tprJ *[tp0621])*. Em 2010 Marra *et al.* aprimoraram o método da tipagem de Pillay *et al.* (1998) com adição de um novo marcador molecular, o gene treponêmico *tp0548*. Essa nova técnica da tipagem chamada de *Enhanced Centers for Disease Control and Prevention* (ECDC) é baseada então na análise das três regiões específicas dos genes *arp, tprEGJ e tp0548* de *T. pallidum*.

Há uma carência de estudos sobre o perfil molecular e de resistência em *T. pallidum* na América do Sul, e no Brasil. Desta forma, objetivou-se desenvolver um diagnóstico molecular para detecção de *T. pallidum* nas amostras clínicas de participantes

com sífilis primária e secundária, bem como caracterizar molecularmente e avaliar o perfil de resistência aos antimicrobianos (azitromicina e doxiciclina) em *T. pallidum*.

2 REVISÃO DE LITERATURA

2.1 Histórico e epidemiologia da sífilis

A origem geográfica do patógeno da sífilis é controversa, sendo explicada por algumas hipóteses. A hipótese Colombiana propõe que o agente causador da sífilis, a espiroqueta *Treponema pallidum* ssp. *pallidum*, surgiu nas Américas, e durante as expedições espanholas em 1492, foi transportada para a Europa pelos marinheiros do explorador italiano Cristóvão Colombo na era genovês (atualmente Itália). Depois, a sífilis se espalhou pelo continente europeu devido à falta de imunidade desta população contra as treponemas (Beale; Lukehart, 2020).

Por outro lado, Majander *et al.* (2020) ao realizar a coleta de amostras de ossos e dentes de indivíduos enterrados na Europa durante os séculos após as expedições de Cristóvão Colombo, identificaram as 4 cepas das treponemas: duas cepas de *T. pallidum* spp. *pallidum*, causadora da sífilis; uma de *T. pallidum* ssp. *pertenue*, causadora da bouba; e uma cepa da treponema que ainda não tinha sido identificada. As cepas treponêmicas antigas foram encontradas na Holanda, Finlândia e Estônia. De forma geográfica, todos esses países estão distantes de onde Colombo e sua tripulação aportaram. O que reforça a hipótese de que as cepas de *T. pallidum* já podiam ter circulado na Europa antes de 1490. Portanto, os dados recentes de sequenciamento genômico de cepas treponêmicas antiga demostra evidências contrárias sobre a hipótese Colombiana (Majander *et al.*, 2020).

Desta forma, a hipótese Pré-Colombiana ou unitária propõe que a sífilis e outras doenças treponêmicas originaram-se na Europa antes do contato com a América, mas não eram identificadas. Portanto, um importante avanço no conhecimento utilizando as técnicas moleculares para demonstrar mais evidências de que a teoria Pré-Colombiana pode ser a mais aceita para explicação do aparecimento de *T. pallidum* na Europa, no entanto mais estudos genéticos são necessários.

No mundo, 7,1 milhões de pessoas foram infectadas com *T. pallidum* em 2020. O diagnóstico precoce e o tratamento eficaz são as principais medidas para controlar a progressão da sífilis (OMS, 2021). No Brasil, segundo dados do Ministério da Saúde os casos de sífilis adquirida tiveram um aumento de 662%, passando de 27.964 (2012) para 213.129 (2022), como observado na figura 1. No período de 2012 a 2022, foram notificados 1.237.027 casos de sífilis adquirida, 537.401 casos de sífilis em gestantes, 238.387 casos de sífilis congênita e 2.153 óbitos por sífilis congênita (Brasil, 2023).

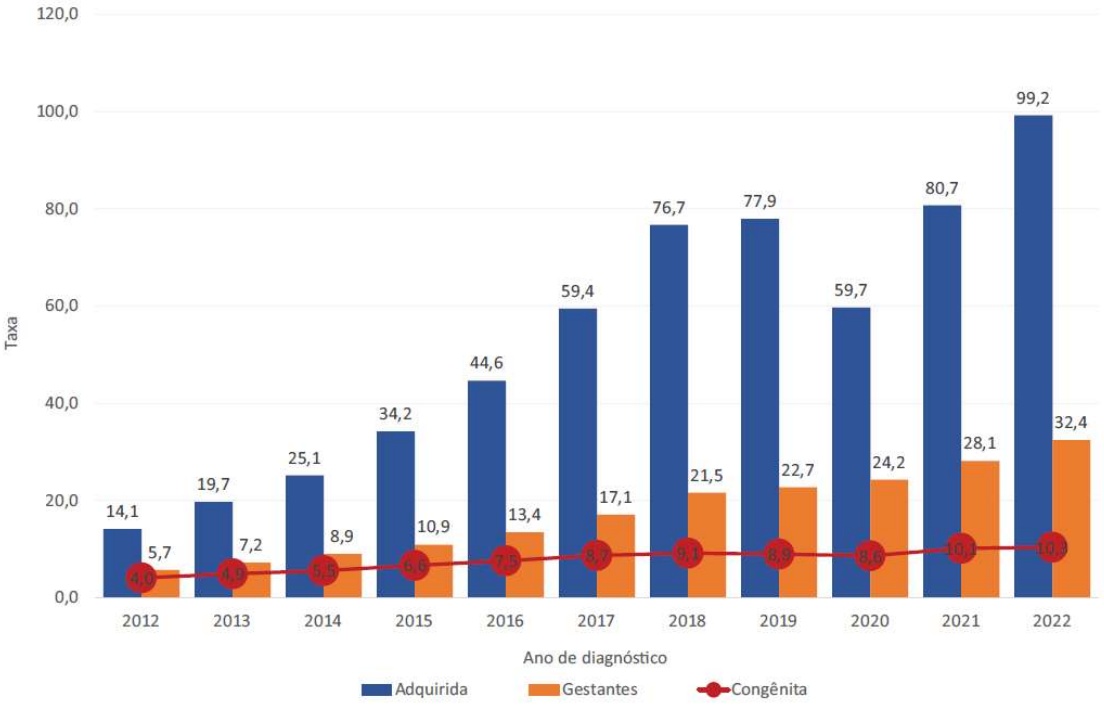

Figura 1. Taxas de detecção de sífilis no Brasil.
Fonte: Boletim Epidemiológico da Sífilis (2023).

Em Mato Grosso do Sul (MS), a sífilis continua a ser um desafio de saúde pública, os dados epidemiológicos revelam uma preocupante tendência de aumento nos casos da doença . Em 2022, foram registrados 2.975 casos de sífilis adquirida, 1.289 casos de sífilis em gestantes e 238 casos de sífilis congênita, com dois óbitos (Brasil, 2023). Estes números são alarmantes e refletem a necessidade urgente de ações preventivas e de intervenção. Um estudo realizado em 2013 em 12 prisões no MS revelou uma prevalência de sífilis de 3,8% entre os privados de liberdade (2% entre os homens; 9% entre as mulheres (Correa *et al.*, 2017), destacando a importância da atenção à saúde em populações vulneráveis. Além disso, estudos mais recentes, como o realizado em Dourados/MS, indicam uma prevalência de 4,4% da infecção por *T. pallidum* em gestantes e 6,04% em mulheres que recebem serviços de saúde primários neste município (Barbosa *et al.*, 2021; Benedetti *et al.*, 2019). Esses dados enfatizam a necessidade de uma vigilância contínua da sífilis.

2.2 Sífilis

A sífilis é uma doença infecciosa multissistêmica e curável, causada pelo *T. pallidum*, uma espiroqueta invasiva e extracelular obrigatória (Ramchandani; Cannon; Marra, 2023). Assim, o *T. pallidum* penetra pelas mucosas ou pele dos pacientes, possuindo também a capacidade de se infiltrar nos gânglios linfáticos regionais e, desta forma, dissemina-se pelo corpo do hospedeiro (Workowski *et al.*, 2021).

O cultivo *in vivo* ou *in vitro* deste patógeno é demorado e oneroso. De fato, essas dificuldades nas técnicas de cultivo, faz com que os estudos epidemiológicos moleculares em largar escala se tornem desafiadores. Além de causar uma limitação na compreensão da atual epidemia da doença, e no conhecimento de *T. pallidum* circulantes no mundo (Edmondson; Hu; Norris, 2018). Em 1998, foi sequenciado o primeiro genoma de *T. pallidum*, denominada de Nichols. Essa cepa foi isolada do líquor de um paciente com neurossífilis e forneceu informações valiosas para identificar fatores de virulência de *T. pallidum*, assim como os potenciais alvos para a tipagem molecular e desenvolvimento de uma vacina (Fraser *et al.*, 1998). O primeiro sequenciamento do genoma completo de *T. pallidum* na China, foi isolada de um paciente com sífilis primária, denominada de Amoy. O seu genoma completo possui aproximadamente 1.139.223 pares de bases (Tong *et al.*, 2017).

A sífilis é clinicamente dividida em estágios ou fases. No seu estágio primário, a principal manifestação clínica é o aparecimento de um ou mais crancos na região genital ou extragenital do paciente, cerca de 10 a 90 dias após o contato infeccioso, decorrente, em sua maioria da transmissão sexual. Os crancos primários, na maioria dos casos, são indolores, o que pode ocasionar a demora do paciente em procurar o atendimento médico. E mesmo sem o tratamento, os sintomas clínicos da fase primária desaparecem com cerca de 45 dias, o que pode levar a falsa percepção de cura pelo paciente (Forrestel; Kovarik; Katz, 2020).

Após o estágio primário, alguns pacientes podem evoluir para o estágio secundário, cujas principais manifestações clínicas são o aparecimento de várias lesões pelo corpo do paciente: boca, região genital e anal, palma das mãos e plantas dos pés, sendo descrita na literatura como a fase mais infecciosa da sífilis (Tudor *et al.*, 2023).

Sem o tratamento, o quadro clínico do estágio secundário desaparece em média após 45 dias. Sendo assim, o paciente permanece assintomático durante anos, denominada de estágio latente recente, com até um ano do estágio secundário, ou latente tardia, após um ano desse estágio (Peeling *et al.*, 2023). Se o paciente não for tratado, o *T. pallidum*

consegue escapar do sistema imunológico do hospedeiro e começam a acometer os órgãos vitais, como coração (Liblik *et al.*, 2023), fígado (Kaya; Kaya, 2020), pulmão (Goda *et al*., 2023), o que leva ao desenvolvimento do último estágio da sífilis no paciente sem o tratamento, denominado de estágio terciário, que pode levar à (Janier *et al.*, 2021).

Quando *T. pallidum* conseguem penetrar no Sistema Nervoso Central do paciente é denominada de neurossífilis. Esta potencial disseminação pode resultar em manifestações neurológicas que ocorrem em qualquer estágio da sífilis, não apenas no terciário (Hamill; Ghanem; Tuddenham, 2023). A figura 2 descreve os principais estágios da sífilis em um paciente não tratado.

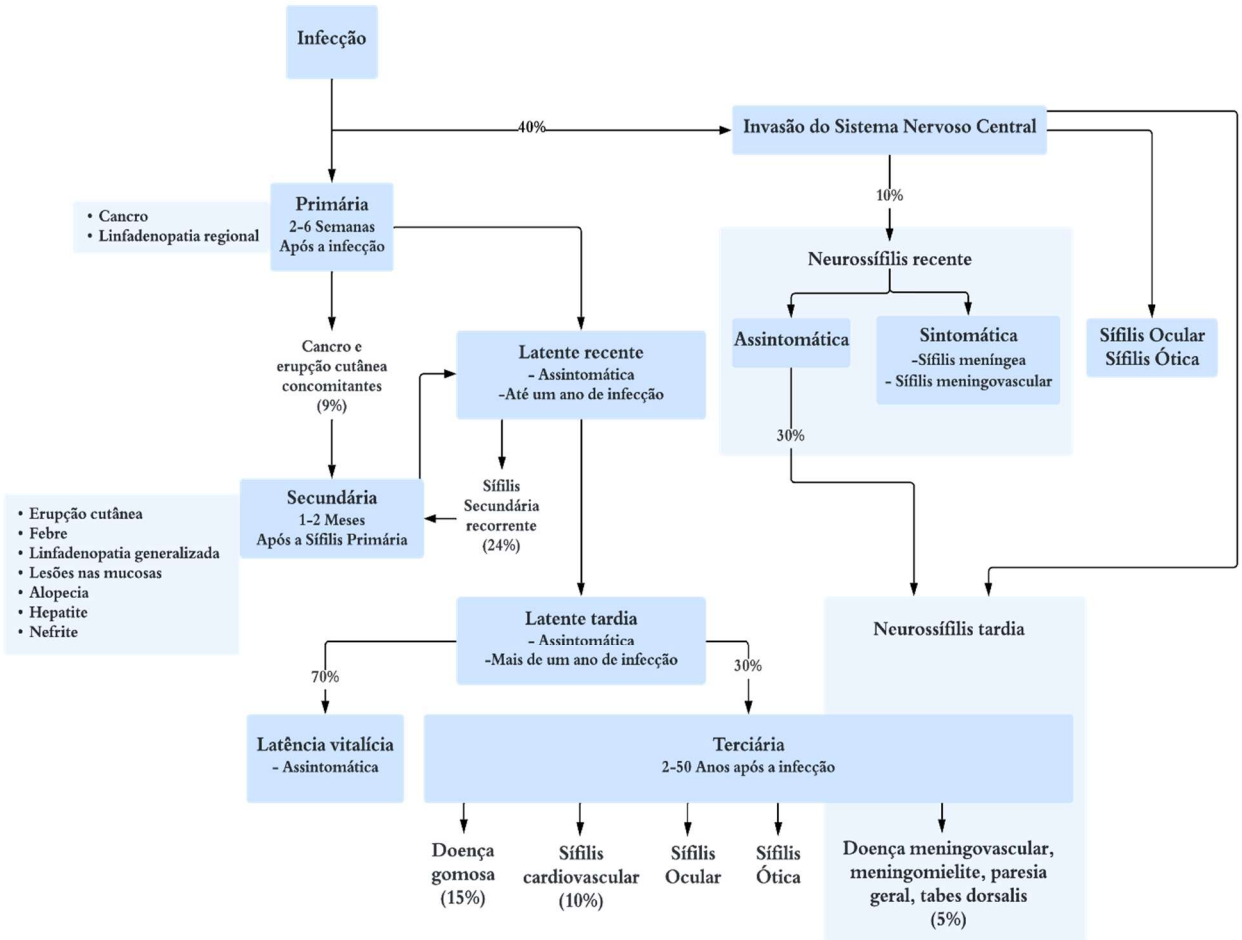

Figura 2. Estágio da sífilis.

Fonte: Modificado de Ghanem; Ram; Rice, 2020.

2.3 Diagnóstico da sífilis

Quando há presença de lesões no paciente com suspeita de sífilis primária ou secundária, o diagnóstico laboratorial é realizado pela identificação de *T. pallidum* nessas lesões. A primeira técnica de detecção do patógeno da sífilis foi a utilização do corante Giemsa modificado por Schaudinn e Hoffmann em 1905 (Kohl; Winzer, 2005). A partir de então as técnicas diretas de diagnóstico para detecção de *T. pallidum* evoluíram, desde a técnica da microscopia em campo escuro (notando a motilidade do microrganismo), para teste utilizando as marcações com prata ou com anticorpos fluorescentes anti-treponema e, recente, com as técnicas moleculares (Whiting; Schwartzman; Khachemoune, 2023). Tais técnicas foram desenvolvidas devido a sensibilidade reduzida dos testes sorológicos para o estágio primário da sífilis, como também a dificuldade na interpretação dos testes sorológicos para diagnóstico e acompanhamento do tratamento (Naidu; Tsang, 2022), é fundamental o desenvolvimento de métodos mais sensíveis para detecção de *T. pallidum*, e contribuir para um diagnóstico mais célere da sífilis.

Os testes sorológicos são um método indireto para o diagnóstico laboratorial da sífilis e são classificados em dois tipos, o teste sorológico treponêmico e não treponêmico (Sato *et al.*, 2022).O teste sorológico treponêmico identifica anticorpos específicos contra *T. pallidum* (Park *et al.*, 2020). O teste sorológico não treponêmico detecta anticorpos não específico, denominados de anticardiolipinas, que são liberados durante o desenvolvimento da infecção das células do hospedeiro e do próprio patógeno (Tuddenham; Katz; Ghanem, 2020).

O teste não treponêmico *Venereal Disease Research Laboratory* (VDRL) detecta anticorpos antilipídios que se formam no homem como resposta imunológica ao material de natureza lipídica, liberado pelas células lesadas no início da sífilis, e do patógeno *T. pallidum* (Gao *et al.*, 2018). O sucesso terapêutico do tratamento da sífilis pode ser acompanhado pela queda gradual da titulação dos anticorpos antilipídios na circulação sanguínea dos pacientes em tratamento. Na sífilis, anticorpos detectados no teste VDRL têm queda gradual quando o tratamento é eficaz, no entanto os anticorpos anti-*T. pallidum*, que permanecem na circulação por cerca de meses a anos, mesmo após a cura do paciente (Tuddenham; Katz; Ghanem, 2020). Wang *et al.* (2021) acompanharam 9 pacientes com sífilis (primária ou secundária) e quantificaram o DNA treponêmico em suas amostras clínicas de saliva após o tratamento. Ao utilizar a técnica molecular, o DNA de *T. pallidum* tornou-se indetectável em média 64 horas após o tratamento dos pacientes (Wang *et al.*, 2021). No entanto, há poucos estudos sobre o acompanhamento do

tratamento do paciente através das técnicas moleculares em todos os estágios da sífilis. Portanto, é fundamental e necessário a realização de mais estudo para uma melhor avaliação da utilização de métodos moleculares para o acompanhamento do tratamento de pacientes com sífilis

2.4 Diagnóstico molecular da sífilis

Desde sua invenção pelo cientista americano Kary Mullis em 1985, a técnica da Reação em Cadeia da Polimerase (PCR, *polymerase chain reaction*) (Mullis, 1990) tem desempenhado um papel importante em diversas áreas do conhecimento científico e se destacado no diagnóstico de doenças infecciosas humanas (Khehra; Padda; Swift, 2023). Com o avanço da biologia molecular, várias técnicas da PCR foram desenvolvidas para se adaptar aos vastos requisitos clínicos e laboratoriais e superar as limitações desta técnica.

Após o sequenciamento do primeiro genoma completo de *T. pallidum* (Fraser *et al.*, 1998), houve um aumento no desenvolvimento das técnicas moleculares para identificação do DNA treponêmico em diversas amostras clínicas de pacientes, em todos os estágios da sífilis. Os marcadores moleculares mais utilizados nos estudos para a detecção do DNA treponêmico em amostras clínicas de pacientes com sífilis são os genes treponêmicos *polA* (Tp0105) e *tpp47* (Tp0574) (Gayet-Ageron *et al.*, 2013) No entanto, outros genes *bmp* e *tp0548* já foram utilizadas em alguns estudos para fins de diagnóstico molecular (Theel; Katz; Pillay, 2020).

Wang *et al.* (2021) conseguiram detectar o DNA de *T. pallidum* em amostras clínicas de saliva e plasma de pacientes em todos os estágios da sífilis. Para isso, foi utilizada a técnica molecular Nested PCR para os genes *polA* e *tpp47* para identificação do DNA treponêmico nas amostras clínicas. E para quantificação do DNA do patógeno foi utilizada a técnica da PCR Digital em Gotas para o *gene polA*. Nas amostras clínicas de saliva, os autores do estudo demostram quantidades elevadas do DNA de *T. pallidum* (Wang *et al.*, 2021), um indicativo de que este substrato pode ser uma via alternativa de transmissão da sífilis. Como também, a utilização da saliva para a detecção de *T. pallidum* com fins de diagnóstico, além da vantagem de ser uma técnica menos invasiva para a coleta.

2.5 Caracterização molecular de *Treponema pallidum*

A tipagem molecular é uma técnica que permite caracterizar o material genético dos microrganismos e pode contribuir na melhoria da vigilância epidemiológica e controle da sífilis (Fu *et al.*, 2020). Além disso, através dessa técnica é possível avaliar a evolução da infecção, monitorar a propagação da sífilis, identificar as principais mutações genéticas que possam influenciar nos fatores de virulência de *T. pallidum*, e a resistência aos antimicrobianos.

Os métodos da tipagem molecular para caracterização de *T. pallidum* tem sido desenvolvidos e aprimorados nos últimos anos, principalmente após 1999, com o sequenciamento da cepa Nichols (Fraser *et al.*, 1998; Fu *et al.*, 2020).A tipagem molecular pode contribui para aprofundar os estudos epidemiológicos de *T. pallidum*, como também monitorar a sua distribuição geográfica e diferenciar entre as treponemas associadas a sintomas clínicos específicos, como cepas de *T. pallidum* mais neuroinvasivas (Marra *et al.*, 2010).

O genoma de *T. pallidum* é conservado e contém regiões polimórficas úteis para a tipagem molecular do patógeno. O primeiro método da tipagem molecular de *T. pallidum* foi desenvolvida por Pillay *et al.* (1998) e denominada de sistema da tipagem do *Centers for Disease Control and Prevention* (CDC). Essa técnica detectou o número de repetições em tandem de 60 pares de base que ocorre no gene da proteína ácida de repetição (arp) (*tp0433*) e analisou o polimorfismo do comprimento do fragmento de restrição (RFLP) dos três genes da família *Tpr* (*T. pallidum repeat*) de *T. pallidum*, os genes tprE (*tp0313*), tprG (*tp0317*) e tprJ (*tp0621*) (Pillay *et al.*, 1998).

Em 2010, na técnica molecular da tipagem CDC, foi adicionada a análise de uma região do gene *tp0548* de *T. pallidum* e foi denominada da tipagem *Enhanced Centers for Disease Control and Prevention* (ECDC) (MARRA et al., 2010). A técnica da tipagem ECDC foi testada em 25 estudos, em 18 países, usando 3014 amostras clínicas. Dessas amostras, em 69,87% (2106/3014) foi possível a tipagem completa da amostra clínica com 167 subtipos identificados (Dai *et al.*, 2012; Fernández-Naval *et al.*, 2019; Flores *et al.*, 2016; Giacani *et al.*, 2018; Grange *et al.*, 2013; Grimes *et al.*, 2012; Kanai *et al.*, 2019; Khairullin *et al.*, 2016; Kojima *et al.*, 2019; Kubanov *et al.*, 2017; Liu *et al.*, 2021; Lu *et al.*, 2017; Mikalová *et al.*, 2013; Mikalová *et al.*, 2017; Peng *et al.*, 2012; Read *et al.*, 2016; Salado-Rasmussen *et al.*, 2016; Sato *et al.*, 2017; Shuel *et al.*, 2018; Vaulet *et al.*, 2017; Venter *et al.*, 2021; Wu *et al.*, 2012; Xião *et al.*, 2016; Yang *et al.*, 2015; Zondag

et al., 2020). O quadro 1 descreve os 19 subtipos mais prevalentes, o que representa 82% dos isolados de *T. pallidum* pelo método ECDC.

A tipagem molecular é mais eficiente em cepas cultivadas ou amostras clínicas de lesões, devido a elevada quantidade de DNA bacteriano específico que pode ser isolado, sem interferência de outros ácidos nucléicos, principalmente, DNA humano das amostras clínicas. Apesar dos recentes avanços, a cultura *in vitro* de *T. pallidum*, a partir das amostras clínicas de pacientes com sífilis, é um desafio e ainda pouco realizada Edmondson; Hu; Norris, 2018).

Quadro 1. Os 19 subtipos de *T. pallidum* mais prevalentes pelo método ECDC de 2106 isolados em 18 países.

	Subtipos ECDC																						
Países	**6d/f**	**11d/f**	**13d/f**	**14a/f**	**14b/f**	**14b/g**	**14d/d**	**14d/f**	**14d/g**	**14e/f**	**14e/g**	**14f/f**	**14f/g**	**14j/g**	**14k/g**	**14l/g**	**14p/g**	**15d/f**	**16d/f**	**Outros***	**Período**	**Referências**
EUA	-	-	-	-	-	-	-	42	57	-	-	-	-	-	-	-	-	11	-	13	2004-2011	Grimes (2012)
Canadá	-	-	-	-	-	2	-	1	36	-	-	-	4	3	2	1	4	-	-	4	2012-2016	Shuel (2018)
Brasil	-	-	-	-	-	-	5	-	6	-	-	-	-	-	-	-	-	-	-	1	2013-2015	Sato (2017)
Argentina	-	2	-	-	-	-	6	6	2	-	-	-	-	-	-	-	-	-	-	5	2006-2013	Vaulet (2017)
Peru	-	-	-	-	-	-	-	3	2	-	-	-	-	-	-	-	-	-	-	2	2013-2014	Flores (2016)
África do Sul	-	1	3	-	2	-	-	26	5	5	-	-	-	-	-	-	-	5	1	47	2010-2018	Venter (2021)
Dinamarca	-	-	-	-	3	7	1	35	107	-	4	3	7	3	2	10	3	-	-	12	2009-2013	Salado-Rasmussen (2016)
França	-	-	-	-	-	-	1	19	49	-	-	-	-	-	-	-	-	1	-	1	2005-2012	Grange (2013)
República Tcheca	-	-	-	-	-	-	-	9	4	1	1	-	-	-	-	-	-	2	-	1	2006-2012	Mikalová (2013)
Bélgica	-	-	-	-	-	-	-	-	1	2	-	-	-	2	-	-	1	-	-	3	2014-2015	Mikalová (2017)
Espanha	-	-	-	1	-	5	-	5	17	-	1	3	9	1	1	1	4	-	-	14	2015	Fernández-Naval (2019)
Itália	1	-	-	2	-	-	-	-	21	-	-	-	-	-	-	-	-	-	-	17	2016-2017	Giacani (2018)
Holanda	-	-	-	-	1	-	5	9	23	3	3	-	1	-	11	-	-	-	-	44	2016-2017	Zondag (2020)
Austrália	-	3	-	-	2	11	2	18	92	1	7	-	2	7	6	6	6	2	-	26	2004-2011	Read (2016)
Japão	-	1	2	-	-	-	-	63	3	1	-	1	-	-	-	-	-	-	-	16	2013-2017	Kojima(2019);Kanai(2019)
China	16	10	31	25	3	-	-	438	2	13	-	-	-	-	-	-	-	43	36	136	2007-2017	Peng(2012);Dai(2012);Xião(2016); Lu(2017);Liu(2021)
Taiwan	-	-	-	5	2	-	-	-	-	2	-	46	-	-	-	-	-	-	-	26	2009-2011	Wu(2012);Yang(2015)
Rússia	-	-	-	-	3	-	-	164	2	-	-	-	-	-	-	-	-	-	-	10	2013-2016	Khairullin(2016);Kubanov(2017)
Total	17	17	38	31	16	25	20	838	429	28	16	53	23	16	22	18	18	66	37	378	2004-2018	25 estudos

*Outros 148 subtipos de *T. pallidum* pelo método ECDC.

Outro método da tipagem molecular independente da cultura de isolados de *T. pallidum* é da tipagem de sequência multilocus (MLST) que permite a identificação de *T. pallidum* e a discriminar as subespécies. A técnica MLST é baseada nas análises das sequências dos genes *tp0136*, *tp0548* e *tp0705*. Para avaliar a estabilidade desses genes como método da tipagem, foi analisada 11 passagens seriadas em coelhos da cepa de referência de *T. pallidum*. E todas as passagens demonstraram o mesmo subtipo, uma estabilidade desses genes da tipagem equivalente a mais de cem gerações treponêmicas (Grillova *et al.*, 2019).

O site pubmlst.org tem acesso aberto que integram dados de sequência populacional com informações de origem e o fenótipo para mais de 100 espécies e gêneros de microrganismos. Desta forma, o banco de dados *Bacterial Isolate Genome Sequence* (BIGSdb, 2010), permite que o site PubMLST inclua todos os níveis de dados de sequência, desde sequências de genes específicos até genomas completos (Jolley; Bray; Maiden, 2018). A frequência de isolados de *T. pallidum* pelo método MLST é demonstrada na figura 3.

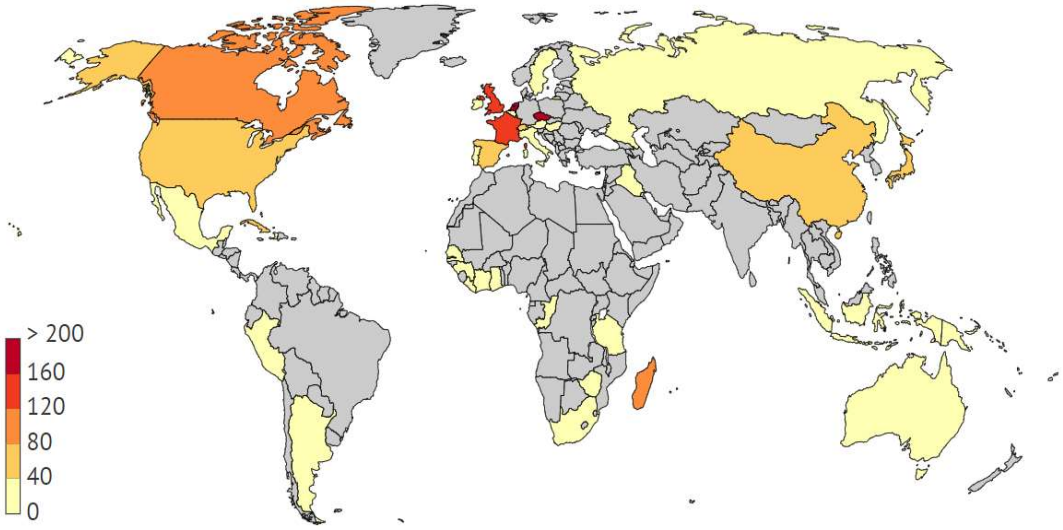

Figura 3. Frequência de subtipos de *T. pallidum* pelo método MLST por países.
Fonte: Jolley et al., 2018.

Em uma metanálise de 43 estudos, a eficiência geral dos métodos da tipagem de *T. pallidum* foi de 71,4% (IC 95%: 63,2–78,9%). As análises de subgrupo indicaram que a eficiência para o método ECDC foi de 72,3% (IC 95%: 60–83,1%) e do método MLST de 67,1% (IC 95%: 61,1–72,7%) (Fu *et al.*, 2020). Um estudo comparando as técnicas ECDC e MLST identificou que a neurossífilis era mais comum em indivíduos infectados com o tipo f do gene *tp0548* (ECDC) ou tipo 2 do gene *tp0705* (MLST) de *T. pallidum*. De fato, representa uma potencial relevância clínica para identificação de pacientes com neurossífilis. Além disso, o custo do método MLST foi o dobro da técnica ECDC (Sahi et al., 2021). Por outro lado, a quantidade de isolados de *T. pallidum* europeus e asiáticos pelo método ECDC e MLST continua superior com os isolados das américas e africanos (Figura 4). Portanto, mais estudo epidemiológicos moleculares no continente americano são necessários para uma melhor compreensão das principais cepas treponêmicas. A técnica ECDC pela sua capacidade discriminatória, estabilidade e custo, desempenha um papel fundamental nos estudos de epidemiologia e manifestações clínicas da sífilis.

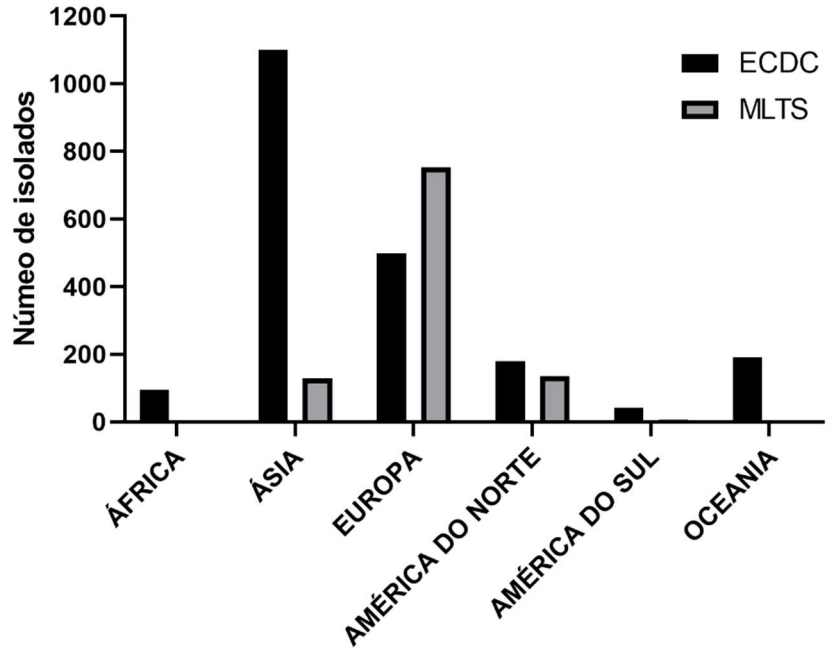

Figura 4. Frequência da tipagem de *T. pallidum* pelos métodos ECDC e MLST por continente.

2.6 Resistência antimicrobiana em *Treponema pallidum*

Os macrolídeos são uma classe de antimicrobianos utilizados para controlar e tratar várias infecções bacterianas humanas. A azitromicina é um macrolídeos utilizado para tratar infecções como pneumonia, sinusite, faringite e amigdalite, bem como infecções sexualmente transmissíveis, tais como as infecções gonocócicas e por clamídia (Patel; Hashmi, 2023). A resistência de *T. pallidum* aos macrolídeos e a falha do tratamento clínico foram relatados em vários países, onde os macrolídeos eram utilizados como um tratamento alternativo para a sífilis. Os estudos indicaram que a mutação A2058G e a mutação A2059G no gene 23S rRNA de *T. pallidum* são mecanismos comuns que causam a resistência aos macrolídeos (Lukehart *et al.*, 2004).

Na China, taxas elevadas de mutação A2058G foram relatadas em Xangai (95,4%), Hunan (97,5%), Shangdong (92,1%) e Xiamen (100%), enquanto a mutação A2059G no gene 23S rRNA de *T. pallidum* só foi relatada em Shangdong (WANG; ABLIZ; DENG, 2023). A resistência de *T. pallidum* aos macrolídeos tem aumentado nos últimos anos em todo o mundo. Em um estudo genômico, a maioria de *T. pallidum* na Austrália (398 [87%] de 456) eram resistentes à azitromicina (TAOUK et al., 2022). A mutação pontual A2058G no gene 23S de *T. pallidum* é a mutação de resistência à azitromicina mais frequente, enquanto a mutação A2059G é incomum como mecanismo de resistência (Li) No entanto, a origem genética e os mecanismos que levam à resistência aos macrolídeos de *T. pallidum* são pouco compreendidos e mais pesquisas são fundamentais e necessárias para uma melhor compreensão. Ainda não foram identificados *T. pallidum* resistentes a penicilina, até o momento o principal antibiótico utilizado para tratamento eficaz da sífilis em gestantes (SALLE et al., 2022; WANG; ABLIZ; DENG, 2023).

3 OBJETIVOS

3.1 GERAL

Realizar o diagnóstico molecular de pacientes com sífilis primária e secundária, bem como caracterizar molecularmente e avaliar o perfil de resistência das amostras clínicas positivas.

3.2 ESPECÍFICOS

Avaliar a prevalência de sífilis entre grupos de risco;

Detectar e quantificar o DNA treponêmico nas amostras clínicas através da Reação em Cadeia da Polimerase (PCR) e da PCR quantitativa em Tempo Real (qPCR) utilizando como marcador molecular, o gene treponêmico *polA*;

Caracterizar molecularmente *T. pallidum* pelo método *Enhanced - Centers for Disease Control and Prevention*;

Investigar o perfil molecular de resistência aos antimicrobianos macrolídeos e as tetraciclinas de *T. pallidum*.

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5 APÊNDICES

Artigo 1: Acta Tropica (Qualis A2)

https://www.sciencedirect.com/journal/acta-tropica/publish/guide-for-authors

Acta Tropica 256 (2024) 107255

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Seroprevalence of *Treponema pallidum* infection among high-risk populations from Brazil

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ARTICLE INFO

Keywords:
Syphilis screening
Syphilis serodiagnosis
Vulnerable populations
Treponemal infections

ABSTRACT

Syphilis is a significant public health concern worldwide. According to the 2020 estimates, nearly 7.1 million new cases of syphilis have been reported globally, with over 30 % of these cases reported from American nations, particularly Brazil. Concerns have been raised regarding the susceptibility of specific groups to syphilis due to challenges and vulnerabilities that place these groups at a higher risk of infections or complications in the treatment outcomes. The present study aimed to compare the seroprevalence and the factors associated with syphilis among such high-risk groups. The study was designed as a cross-sectional one and was conducted with pregnant women, people living with HIV (PLHIV), people living with tuberculosis (PLTB), indigenous and healthy populations in Mato Grosso do Sul, Brazil. The study was conducted between June 2019 and August 2022, during which the included patients were subjected to treponemal and non-treponemal serological assays. The study also included a survey conducted through a self-reported questionnaire to collect information regarding the participants' demographics and sexual behaviors. A total of 550 samples were collected, with 110 participants in each of the five groups. The results of the study revealed that the seroprevalence of *Treponema pallidum* infection in pregnant women, PLHIV, PLTB, indigenous and healthy populations of the study region was 10 % ($n = 11/110$), 41.81 % ($n = 46/110$), 17.27 % ($n = 19/110$), 5.45 % ($n = 6/110$), and 8.18 % ($n = 9/110$), respectively. Homosexual orientation ($p = 0.04$) and a history of sexually transmitted infection (STI) ($p = 0.01$) were associated with the seroprevalence of *T. pallidum* infection in PLHIV. However, no such associations were noted in the remaining four groups. The seroprevalence of *T. pallidum* infection was observed to vary significantly among the different high-risk groups, which highlighted the persistent concern of syphilis, particularly among vulnerable populations. These findings underscore the significance of focused interventions and public health strategies customized to the specific requirements of each of the groups evaluated in the present study to decrease the number of cases of syphilis and thereby prevent future complications in patients with other serious infections.

1. Introduction

Syphilis is a sexually transmitted infection (STI) caused by *Treponema pallidum* and is associated with substantial morbidity and mortality. *T. pallidum* is transmitted through sexual contact with infectious lesions, which may happen either through blood transfusion or from a pregnant woman to her fetus. In 2020, approximately 7.1 million new cases of syphilis were reported worldwide. The World Health Organization (WHO) aims to reduce the prevalence of syphilis by 90 % and eliminate congenital syphilis by the year 2030 (World Health Organization, 2021). Since 2010, a continual increase has been recorded in the global spread of syphilis. Several high-income nations, such as Australia

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https://doi.org/10.1016/j.actatropica.2024.107255
Received 20 March 2024; Received in revised form 26 April 2024; Accepted 15 May 2024
Available online 16 May 2024

(Phakey et al., 2024), European nations (Mitjà et al., 2023), Japan (Mukherjee et al., 2023), and the United States (Htet et al., 2023), are currently experiencing a resurgence of syphilis. In Brazil, the number of new cases of syphilis has increased by 662 %, rising from 27,964 cases reported in 2012 to 213,129 cases in year 2022 (Brasil, 2023).

This alarming increase in the new cases of syphilis has emerged as a significant concern for public health systems, necessitating a comprehensive and effective response to contain the spread of this disease and ensure access to proper treatment for the affected patients. The spread of syphilis underscores the importance of education on sexual and reproductive health and that of promoting condom usage and regular testing for early detection and timely treatment (Tao et al., 2023). Moreover, when left untreated, syphilis could lead to severe neurological symptoms (Wu et al., 2024). Untreated syphilis during pregnancy could lead to miscarriage, stillbirth, maternal and infant morbidity, and infant death (Stafford et al., 2024).

Therefore, it is crucial to monitor *T. pallidum* infection, particularly in vulnerable populations, which include pregnant women, people living with HIV (PLHIV), people living with tuberculosis (PLTB), and indigenous populations. Such groups often encounter unique challenges and vulnerabilities that increase their risk of infection or complication during treatment (Odendaal and Smit, 2022; Shorer et al., 2023). Therefore, screening tests are essential for detecting *T. pallidum* infection in this populations. Unfortunately, only a few comprehensive estimates of the prevalence in these high-risk populations are available currently (Zhang et al., 2022). Brazil has a huge indigenous population and also PLHIV and PLTB in significant numbers (Schnaufer et al., 2023; Vaz et al., 2023). Accordingly, targeted healthcare interventions and public health strategies become further important to address the specific requirements and challenges of these populations. In this context, the present study aimed to investigate the seroprevalence of *T. pallidum* infection among the four high-risk populations in Dourados, Mato Grosso do Sul (MS), and assess the risk factors associated with this infection.

2. Material and methods

2.1. Study design and study populations

The present study was designed as a cross-sectional study, which was conducted between June 2019 and August 2022 in Dourados, MS, the Midwestern region of Brazil. The case-control study compared four risk groups for *Treponema pallidum* infection with a control group in a 1:1:1:1 ratio. Dourados has an estimated population of 243.367 residents, which was categorized mainly into five groups for the present study: pregnant women, people living with HIV (PLHIV), people living with tuberculosis (PLTB), indigenous population, and healthy population. Individuals from the indigenous community of Dourados were included in the study. The eligibility criteria for inclusion were as follows: residence in Dourados; pregnancy; a confirmed diagnosis of HIV or TB; indigenous person. The sample size for the study was calculated based on approximately 243.367 adults and an expected *T. pallidum* infection prevalence of 1.11 %, with an accuracy of 1 % and a confidence interval of 95 %, which revealed that a minimum of 427 samples were required. In order to compensate for the potential decrease in the number of participants due to refusals to provide consent, an additional 20 % of the total participants were included. Written informed consent was obtained from all selected participants.

2.2. Data and blood sample collection

Convenience sampling was adopted for all selected participants and included an interview and providing a biological sample. The study was conducted in two stages, as described ahead: 1) Interviews were conducted using a standardized questionnaire to collect data on demographics and sexual behaviors. The indigenous participants self-reported their villages (Bororó and Jaguapirú) and ethnic groups (Guarani and Kaiowa). The responses for categorical variables were recorded as either "Yes" or "No," while those for the numerical variables were grouped into categories. 2) Blood samples were collected for syphilis serology. Medical guidelines were followed, and adequate antiseptic measures were maintained while collecting 3 mL of the peripheral venous blood sample from each participant using a vacuum tube system. Each sample was then processed to separate the contents from the serum. The serum samples were finally stored at −20 °C.

2.3. Diagnostic tests

Rapid treponemal (RT) tests were conducted using the point-of-care test (POCT) (ABON Biopharm, Hangzhou, China) sample screening. The reactive samples in the Venereal Disease Research Laboratory (VDRL) test (Abbott Murex, Dartford, UK) were serially diluted and titrated to detect anticardiolipin antibodies. The non-reactive serum samples in the VDRL test were subjected to enzyme-linked immunosorbent assay (ELISA) (ICE* Syphilis, DiaSorin, Saluggia, Italy) to detect anti-*T. pallidum* IgG and IgM. *T. pallidum* infection positivity was defined as the sample exhibiting reactivity in both treponemal and non-treponemal tests Laboratory tests were conducted at the Laboratory of Health Sciences Research (LPCS/UFGD) following the manufacturer's instructions. Each participant received the results of their serological tests, and a physician specializing in infectious diseases prescribed the appropriate treatment for the syphilis-positive patients.

2.4. Statistical analysis

The data from the questionnaire survey and serological tests were collected, and after subsequent validation, were submitted to the Research Electronic Data Capture (REDCap) y database. The data were analyzed using the SPSS version 29.0 software package (SPSS Inc., Chicago, IL, USA). The association between syphilis serology and variables was assessed using Chi-squared tests. Bivariate logistic regression analyses were conducted to compare the results of syphilis serology obtained for the different groups, namely pregnant women, PLHIV, PLTB, and the indigenous population, with those obtained for the health population. Syphilis serology was used as the binary dependent variable, and the various potential risk factors were used as independent variables. The statistical significance threshold was set to $p < 0.05$. The odds ratios (ORs) with 95 % confidence intervals (95 % CI) were determined using logistic regression.

2.5. Ethical aspects

The study was approved by the National Research Ethics Commission (number 2.000.496, 5.588.205, 1.402.529 and 2.195.047).

3. Results and discussion

The present study included individuals aged 18 years or older who provided written informed consent for participation in the study and were residents of Dourados/MS. A total of 550 samples were included in the study, with 110 participants in each of the five groups. The median age of the study participants was 34.5 years (Table 1). The overall seroprevalence of *T. pallidum* infection the healthy population was 8.18 % ($n = 9/110$). The study involved four high-risk subgroups: pregnant women, PLHIV, PLTB, the indigenous populations, and the estimated seroprevalence rates in these subgroups were 10 % ($n = 11/110$), 41.81 % ($n = 46/110$), 17.27 % ($n = 19/110$), and 5.45 % ($n = 6/110$), respectively (Fig. 1).

The results of the multivariate analysis suggested that individuals with a history of sexually transmitted infections (STIs) were at a higher risk of acquiring syphilis during pregnancy, as corroborated by an odds ratio (OR) of 63.5 [95 % confidence interval [CI]: 15.3–262.4)]. Similar

Table 1
Sociodemographic and risk behavior characteristics of population of this study.

Variables	Health $N = 110$	Pregnant $N = 110$	PLHIV $N = 110$	PLTB $N = 110$	Indigenous $N = 110$
	Number (Percentage)				
Median age [IQR]	37 [26.75, 54]	26 [21, 31]	43 [32, 52]	33.5 [28, 44.75]	33.5 [25, 44]
Level of education					
Illiterate	1 (0.9)	2 (1.8)	2 (1.8)	3 (2.72)	15 (13.63)
Basic education	32 (29.1)	59 (53.63)	54 (49.08)	81 (73.62)	74 (67.30)
High school	45 (40.9)	33 (29.99)	40 (36.36)	15 (13.63)	15 (13.63)
Graduation	32 (29.1)	16 (14.58)	14 (12.72)	6 (5.45)	3 (2.72)
Ethnicity					
White	48 (43.63)	46 (41.81)	42 (38.17)	30 (27.27)	0 (0)
Mixed	50 (45.5)	48 (43.63)	44 (39.99)	57 (51.81)	0 (0)
Black	8 (7.27)	6 (5.45)	19 (17.27)	13 (11.83)	0 (0)
Indigenous	1 (0.90)	7 (6.36)	0 (0)	10 (9.09)	110 (100)
Asian	2 (1.8)	3 (2.75)	5 (4.54)	0 (0)	0 (0)
Sexual and behavioral history					
Stable partner	36 (32,72)	85 (77.26)	30 (27.27)	37 (33.63)	85 (74.53)
Multiple sexual partners	70 (63,63)	22 (19.99)	73 (66.35)	48 (43.63)	27 (24.54)
History of STI (s)	16 (14.58)	13 (11.83)	24 (21.81)	3 (2.75)	1 (0.9)
Syphilis serologic positive	9 (8.18)	11 (10)	46 (41.81)	19 (17.27)	6 (5.45)

IQR, Interquartile range; PLHIV, People living with HIV; PLTB, People living with tuberculosis; STI(s), sexually transmitted infections.

results were observed for PLHIV with an odds ratio (OR) of 7.20 (95 % CI: 2.73–18.99), PLTB with an OR of 5.21 (95 % CI: 1.23–21.97), and indigenous populations with an OR of 5.21 (95 % CI: 1.23–21.97), as presented in Table 2.

The rates of syphilis worldwide, in both adults and congenitally, have significantly increased in the last decade despite public health efforts to contain the spread of the disease. Penicillin shortages, inadequate screening, and substandard treatment have all contributed to the global burden of disease. The results of this study, which included a total of 550 samples from five distinct groups, provide valuable insights into the prevalence of *T. pallidum* infection in both health and high-risk populations. The median age of participants across all groups was 34.5 years, indicating a diverse range of age demographics, with ages ranging from 26 to 43 years among the groups.

The study reported an overall seroprevalence of *T. pallidum* infection of the 8.18 % (n = 9/110) in the healthy population, indicating the expressive rate of infection even among individuals outside traditionally considered high-risk groups. Comparing these findings to global and regional prevalence rates provides additional context. In 2020, the worldwide seroprevalence of *T. pallidum* infection was 0.6 % (0.5–0.7 %) (World Health Organization, 2021). The WHO European Region had the lowest seroprevalence of *T. pallidum* infection among all WHO regions for both genders, with a syphilis rate of 0.11 % (0.09–0.13) (Mitjà et al., 2023). These statistics highlight the comparatively higher prevalence of syphilis observed in the studied population compared to global and regional averages, emphasizing the need for targeted interventions and public health strategies to address the specific challenges of syphilis transmission within the community studied.

Our study showed that the seroprevalence of *T. pallidum* infection varies significantly among different high-risk groups. It is important to note that pregnant, PLHIV, and PLTB were found to have higher rates of infection compared to the indigenous and healthy populations. Although the seroprevalence of *T. pallidum* infection among the groups studied was lower in indigenous people, the prevalence identified in our study is higher than that reported in Latin American indigenous populations (Russell et al., 2019). The cross-sectional study among indigenous populations in the Brazilian Amazon reported the prevalence of syphilis of 1.82 % (Benzaken et al., 2017). These findings emphasize the importance of targeted interventions and tailored public health strategies to address the specific needs and challenges faced by indigenous populations regarding syphilis prevention, screening, and treatment. Efforts to control *T. pallidum* transmission should consider the unique circumstances and contexts of these populations to achieve equitable health outcomes and reduce disparities in the burden of syphilis. The differences in prevalence rates highlight the variability of syphilis epidemiology within populations and underscore the need for context-specific research and interventions. Factors such as geographical location, cultural practices, access to healthcare services, and socioeconomic conditions may contribute to variations in prevalence rates among different population as well as, indigenous communities.

The seroprevalence of *T. pallidum* infection in pregnant women in our study is higher than the global average of 0.8 %, with variations ranging from 0.7 % to 0.9 %, and low-income countries with 3.3 % (2.2–4.6 %) (Wu et al., 2023). In addition, the impact of syphilis in pregnant women can be significant, affecting both the mother and the developing fetus.

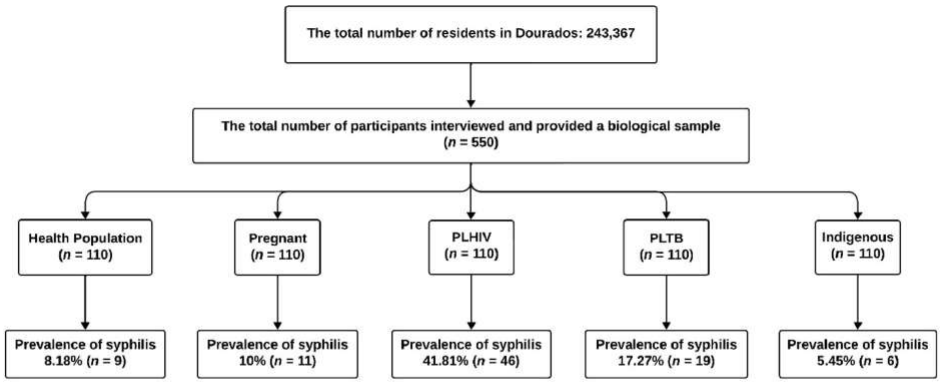

Fig. 1. Flow chart of populations and serologic results of this study.

Table 2
Variables associated with seroprevalence of *T. pallidum* infection in population of this study.

Variables	Pregnant			PLHIV			PLTB			Indigenous		
	χ^2 *P*-value	OR (95 % CI)	*P*-value	χ^2 *P*-value	OR (95 % CI)	*P*-value	χ^2 *P*-value	OR (95 % CI)	*P*-value	χ^2 *P*-value	OR (95 % CI)	*P*-value
Stable partner	0.637	0.56 (0.12–2.55)	0.457	0.610	0.67 (0.25–1.76)	0.423	0.102	0.46 (0.13–1.69)	0.249	0.050	0.24 (0.02–2.14)	0.203
Number of sexual partners	0.141	18.30 (0.58–573.29)	0.098	0.130	4.10 (0.49–34.26)	0.192	0.067	9.37 (0.57–153.75)	0.117	0.125	4.37 (0.66–28.83)	0.125
Level of education	0.141	0.18 (0.003–9.47)	0.398	0.314	1,042,444 (0.000)	0.999	0.502	0.40 (0.06–2.51)	0.333	0.383	0.51 (0.08–3.02)	0.459
Family income	0.826	1.46 (0.39–5.52)	0.571	0.097	0.69 (0.30–1.61)	0.401	0.634	1.90 (0.53–6.79)	0.322	0.391	1.63 (0.54–4.91)	0.385
Homosexual orientation	0.163	12.16 (1.09–135.09)	0.042	0.000	3.88 (1.56–9.68)	0.004	0.049	6.32 (0.95–41.99)	0.056	0.317	1.63 (0.57–4.63)	0.357
Diabetes	0.942	2.35 (0.25–21.67)	0.448	0.549	1.16 (0.18–7.28)	0.871	0.602	1.22 (0.06–24.4)	0.893	0.942	1.15 (0.19–7.01)	0.873
High blood pressure	0.151	4.13 (0.76–22.48)	0.100	0.180	1.85 (0.66–5.13)	0.235	0.105	4 (0.46–34.10)	0.205	0.675	4.89 (0.73–32.66)	0.101
Obesity	0.434	0.66 (0.06–6.75)	0.728	0.469	0.55 (0.12–2.38)	0.428	0.897	0.36 (0.01–10.97)	0.564	0.574	0.45 (0.02–7.19)	0.577
History of STIs	<0.001	63.57 (15.39–262.47)	<0.001	0.000	7.20 (2.73–18.99)	<0.001	0.004	6.83 (1.26–36.78)	0.025	0.063	5.21 (1.23–21.97)	0.025
Other health problems	0.526	0.41 (0.06–2.52)	0.343	0.194	0.59 (0.12–2.93)	0.524	0.925	0.44 (0.03–5.59)	0.528	0.701	0.98 (0.18–5.25)	0.983

PLHIV, People living with HIV; PLTB, People living with tuberculosis; STI(s), sexually transmitted infections.

This underscores the importance of comprehensive prenatal care, routine screening during pregnancy, early detection, and prompt treatment to prevent adverse outcomes for both the mother and the baby (Stafford et al., 2024). Effective public health interventions aimed at preventing and controlling *T. pallidum* transmission in pregnant women are essential for reducing the burden of congenital syphilis and improving maternal and child health outcomes.

Notably, the multivariable analysis showed an association between seroprevalence of *T. pallidum* infection and a recent history of STIs, with a probability value of 0.04. This result highlights the importance of education on sexual and reproductive health, as well as promoting the use of condoms and regular testing for early detection and appropriate treatment (Tao et al., 2023). *T. pallidum* infection may be increased the risk of acquiring HIV. Syphilis can cause genital ulcers and mucosal lesions, which provide a portal of entry for HIV transmission during sexual contact (Wu et al., 2021). Additionally, the inflammatory response triggered by syphilis may increase the concentration of immune cells susceptible to HIV infection in genital tissues, further facilitating HIV acquisition. Moreover, the presence of syphilis can lead to disruptions in the genital mucosal barrier, potentially enhancing the likelihood of HIV transmission (Ruangtragool et al., 2022).

Furthermore, individuals with PLTB are also at higher risk of acquiring syphilis. The compromised immune system in PLTB patients makes them more susceptible to acquiring various infections, including *T. pallidum* (Brett et al., 2020). Additionally, factors such as social determinants of health, including poverty, homelessness, and substance abuse, which are commonly associated with TB, may also increase the risk of engaging in behaviors that facilitate *T. pallidum* transmission (Craig and Zumla, 2015). Moreover, the presence of syphilis can exacerbate the clinical course of both HIV and TB infections. Co-infection with *T. pallidum* in PLHIV has been associated with higher viral loads, lower CD4 cell counts, and increased risk of HIV transmission to uninfected partners. Similarly, in PLTB patients, *T. pallidum* infection co-infection has been linked to delayed TB diagnosis, increased severity of TB symptoms, and poorer treatment outcomes (Fan et al., 2021). Thus, the evidence from studies suggests that syphilis serves as a significant risk factor for both HIV and TB infections. Understanding and addressing the interplay between these infections is crucial for comprehensive healthcare management, particularly in populations at heightened risk for these diseases. Efforts to control *T. pallidum* transmission should be integrated into broader HIV and TB prevention and treatment programs to effectively mitigate the impact of these co-infections on public health.

Despite providing valuable insights into the seroprevalence of *T. pallidum* infection and associated risk factors, the study has limitations that may impact the interpretation of the findings. Firstly, the findings may not be representative of other regions in Brazil, and risk factors for *T. pallidum* infection can vary by location, which limits the generalizability of the results to a broader context. Secondly, utilizing a self-reported questionnaire to collect demographic and sexual behavior information may introduce the potential for recall bias. Furthermore, other demographic factors, such as socioeconomic status, education level, and access to healthcare, were not thoroughly examined. These factors could influence the seroprevalence of *T. pallidum* infection and should be considered in a more comprehensive analysis.

In conclusion, the high prevalence rates observed underscore the urgent need for continued surveillance, awareness campaigns, and collaborative efforts to control and eventually eradicate syphilis. This study provides valuable insights that can guide the development of public health policies aimed at reducing *T. pallidum* transmission and enhancing the overall health outcomes of diverse and at-risk populations. However, a longitudinal study involving other geographical regions would offer a more comprehensive understanding of the dynamics of syphilis infection within Brazilian populations.

Funding information

This work was partially supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico, Fundação de Apoio ao do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Universidade Federal da Grande Dourados (UFGD). JHFSQ received a scholarship from FUNDECT. EVOP and SS from CNPq. GHAS received a CNPq junior postdoctoral fellowship through the FUNDECT (76/2023). The funding agencies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

CRediT authorship contribution statement

Júlio Henrique Ferreira de Sá Queiroz: Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis. **Marcelo dos Santos Barbosa:** Resources, Methodology, Investigation, Conceptualization. **Emily Vitória de Oliveira Perez:** Resources, Investigation. **Bruna Oliveira da Silva:** Resources, Investigation. **Gleyce Hellen de Almeida de Souza:** Writing – review & editing, Resources. **Crhistinne Cavalheiro Maymone Gonçalves:** Writing – review & editing, Data curation. **Julio Croda:** Writing – review & editing, Data curation. **Simone Simionatto:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Artigo 2: Brazilian Journal of Microbiology (Qualis A2)

https://link.springer.com/journal/42770/submission-guidelines

Brazilian Journal of Microbiology
https://doi.org/10.1007/s42770-024-01342-w

BACTERIAL, FUNGAL AND VIRUS MOLECULAR BIOLOGY - SHORT COMMUNICATION

Typing of *Treponema pallidum* in a Brazilian sample and follow-up of treatment using molecular assays

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Received: 2 August 2023 / Accepted: 8 April 2024

Abstract

Syphilis remains a significant public health concern, with serological assays being the primary method for diagnosis. However, molecular techniques have proven to be reliable tools for the diagnosis and understanding of the transmission dynamics of *Treponema pallidum* infection. This study aimed to evaluate the efficacy of syphilis treatment using molecular assays, perform Enhanced Centers for Disease Control and Prevention (ECDC) typing, and analyze resistance (macrolide and doxycycline) in the *T. pallidum* isolate. PCR assay amplified treponemal DNA only from the lesion sample, whereas qPCR was able to amplify DNA in both lesion and blood samples before treatment. Throughout the treatment follow-up, qPCR effectively did not identify treponemal DNA in the blood for up to one to two weeks after treatment. ECDC typing revealed the genotype 14 e/g in the Brazilian *T. pallidum* isolate, and the presence of the A2058G mutation in 23 S rRNA gene, indicating macrolide resistance. Although, the G1058C mutation in 16 S rRNA gene was not detected. Notably, qPCR demonstrated its potential for diagnosing *T. pallidum* in blood samples, even when the treponemal DNA levels were low, enabling more accurate and sensitive diagnosis and guiding better syphilis therapy. In addition, to the best of our knowledge, this study represents the first identification of subtype 14 e/g and azithromycin resistance in a Brazilian *T. pallidum* isolate.

Keywords Syphilis · qPCR · ECDC typing · Treatment · Resistance

Introduction

Infection caused by *Treponema pallidum* is a global public health problem. The transmission of syphilis can occur through sexual or hematogenous pathways, including mother-to-child transmission [1]. The evolution of this infection presents stages (primary, secondary, latent, and tertiary), each associated with different signs and symptoms, making its diagnosis and treatment follow-up a challenge [2].

Currently, the diagnosis of syphilis is divided into direct and indirect methods. Direct detection of *T. pallidum* is achieved through techniques like darkfield microscopy or direct fluorescent antibody testing when lesions are present [3]. Indirectly, the treponemal antibody test becomes reactive around 10–15 days after the ulcer (chancre) evolves and may not differentiate active and previously treated syphilis. Nontreponemal (antiphospholipid) antibody tests, such as Venereal Disease Research Laboratory (VDRL) or Rapid Plasma Reagin (RPR), are employed to monitor syphilis treatment, with higher titers potentially correlating with disease activity [4]. Despite the variety of detection methods, those technics' low sensitivity and/or specificity can limit syphilis diagnosis.

In order to overcome these limitations, nucleic acid amplification tests such as Polymerase Chain Reaction (PCR) or real-time quantitative PCR (qPCR) have been developed and proven effective in detecting *T. pallidum* in

Responsible Editor: Fernando R. Pavan.

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Published online: 15 May 2024

various clinical specimens and all stages of syphilis. These molecular techniques are mainly valuable in lesions of early infection where the serological tests may still yield nonreactive results [5]. Moreover, molecular typing has been useful to understand the dynamic transmission of the *T. pallidum* infection. The Enhanced Centers for Disease Control and Prevention (ECDC) typing scheme has emerged as a molecular tool for determining the diversity of circulating *T. pallidum* strains in different countries [6].

Considering these advancements, this study aimed to identify *T. pallidum* in a Brazilian patient with syphilis using both lesion and blood samples, through PCR and qPCR assays, as well as to monitor the efficacy of syphilis treatment. Furthermore, the ECDC typing system was used for the characterization of *T. pallidum* isolate, and an analysis of mutations associated with antibiotic resistance in the 23 S rRNA gene and the 16 S rRNA gene was conducted.

Methods

Study design

The sample collection was carried out with participants from Dourados (Supplementary Fig. 1). Dourados, Mato Grosso do Sul (MS), is located in the Central-West region of Brazil, which has a total population of 2,756,700 and is situated in the Midwest region of Brazil. Dourados has a Gross Domestic Product per capita of USD$10,935.74, and the city's infant mortality rate stands at 12.71 per 1,000 live births [7]. In August 2016, a female was admitted to a gynecological outpatient clinic in a tertiary hospital, complaining of pain in the genital area, vaginal discharge, episodes of unmeasured fever for two days, pruritus and a vaginal lesion that had persisted for approximately one month. During the gynecological examination, an ulcerated lesion of approximately 2 cm was observed on the external part of the genitalia, and the vaginal canal showed hyperemia and whitish secretion.

Serological assay

To perform serological and molecular assays, 10 mL of peripheral venous blood and a swab of lesion exudate were collected. The point-of-care test (POCT) for syphilis (ABON Biopharm, Hangzhou, China), the enzyme-linked immunosorbent assay (ELISA) for detection of anti-*T. pallidum* IgM and IgG antibodies (ICE Syphilis, DiaSorin, Saluggia, Italy), and the VDRL test (Abbott Murex, Dartford, UK) were conducted by the manufacturer's instructions.

DNA extraction

The genital lesion was swabbed directly to obtain a serous exudate, which was then suspended in 600 µL of phosphate-buffered saline, following a previously described procedure [8]. DNA extraction from the lesion and whole blood samples was performed using the QIAamp DNA Blood Mini kit (Qiagen, Germantown, Maryland, USA) following the manufacturer's protocol. The extracted DNA was subsequently quantified using a BioDrop DUO spectrophotometer (BioDrop, Cambridge, UK).

T. pallidum DNA detection

The *polA* (*tp0105*) gene was amplified by PCR and qPCR assays. Specific sets of primers (Table 1) were used for these amplification processes. The PCR assay, along with negative and positive controls was performed following a previously described procedure. To assess the integrity and sufficiency of the extracted DNA, a PCR test targeting the human β-globin gene was conducted [9].

Table 1 Sequence primers used in PCR, qPCR, molecular typing, and resistance

Primers	Nucleotide sequence	Application
polA Forward	5' TGCGCACGAAGATAGTGTGT 3'	*polA* gene
polA Reverse	5'AGCAGACGTTACATCGAGCGGA 3'	
tp0548 Forward	5' GGTCCCTATGATATCGTGTTCG 3'	Molecular typing
tp0548 Reverse	5' GTCATGGATCTGCGAGTGG 3'	
arp Forward	5' ATCTTTGCCGTCCCGTGTGC 3'	
arp Reverse	5' CCGAGTGGGATGGCTGCTTC 3'	
EGJ forward 1	5' ACTGGCTCTGCCACACTTGA 3'	
EGJ Reverse 1	5' CTACCAGGAGAGGGTGACGC 3'	
EGJ forward 2	5' CAGGTTTTGCCGTTAAGC 3'	
EGJ Reverse 2	5'AATCAAGGGAGAATACCGTC 3'	
23 S Forward	5' GTACCGCAAACCGACACAG 3'	Resistance Macrolide
23 S Reverse	3' AGTCAAACCGCCCACCTAC 5'	
16 S Forward 1	5' GTGGATGAGGAAGGTCGAAA 3'	Resistance Tetracycline
16 S Reverse 1	3' CAGAGTCCCCAACACCACTT 5'	
16 S Forward 2	5' TCAACTTGGGAACTGCACTG 3'	

The qPCR targeting the *polA* gene was performed using the SYBR Green JumpStart Taq Ready Mix (Sigma-Aldrich, MO, USA). The qPCR reagents included 2x SYBR Green Master Mix, 250 nM of each primer, and 2 µL of the isolated total DNA, resulting in a final volume of 25 µL. The cycling conditions were 94 °C for 2 min, followed by 45 cycles of 94 °C for 15 s, and 60 °C for 1 min. Subsequently, a melting step was performed with a temperature increase from 65 to 95 °C at a rate of 0.5 °C/s. The amplification and melting curve analysis were carried out using the Bio-Rad CFX Manager software version 3.0.1224 (Bio-Rad Laboratories, Inc.).

Molecular typing of *T. pallidum*

The ECDC typing scheme of *T. pallidum* was determined based on the number of 60-bp repeats copies of the *arp (tp0433)* gene, following previously described methods [10]. The amplification and analysis of the *tprE (tp0313), tprG (tp0317), tprJ (tp0621)*, and *tp0548* genes were conducted as described in previous studies [11]. The primers used in the molecular typing system are described in Table 1.

Resistance analysis to azithromycin (macrolide) and doxycycline (tetracycline)

The PCR assay to assess resistance to azithromycin and doxycycline from *T. pallidum* was based on the amplification of the 23 S rRNA and 16 S rRNA genes, respectively, using primers described in Table 1. Sanger sequencing was performed to detect the A2058G and A2059G mutations of the 23 S rRNA gene, and G1058C point mutation of 16 S rRNA gene [10]. The obtained sequences were analyzed using the BioEdit software (Version 7.2 available from https://bioedit.software.informer.com/7.2/).

Table 2 PCR, qPCR, and VDRL results of the clinical samples analyzed in this study

	PCR		qPCR		VDRL TITER
TREATMENT	LESION	BLOOD	LESION	BLOOD	SERUM
BEFORE	+	-	+	+	1:128
ONE WEEK	*	-	*	-	1:64
TWO WEEKS	*	-	*	-	1:64
THREE MONTHS	*	NS	*	NS	1:16

Note. '*': Lesion absence; '+': Positive; '-': Negative; 'NS': No sample

Results

The medical diagnosis for primary syphilis was completed based on the patient's clinical presentation and serologic results. The serum showed reactivity for the POCT for syphilis, ELISA, and the VDRL with a titer of 1:128. Subsequently, the treatment plan was initiated, consisting of a single intramuscular dose of 1 gram ceftriaxone (IM) and weekly intramuscular injections of penicillin G benzathine, totaling 2.4 million units during three weeks. Before treatment, and also one and two weeks after the beginning of treatment, blood samples were collected for *T. pallidum* DNA extraction.

In our study, DNA from the treponemal bacterium was only detected in the primary-stage lesion using PCR. However, this molecular technic could not detect *T. pallidum* DNA in whole blood samples before and after treatment. Conversely, qPCR detected copies of the *T. pallidum* DNA in the lesion and blood samples before treatment. Furthermore, treponemal DNA was not identified in the lesion or blood samples one and two weeks after treatment. In the positive control, our primer set for the *polA* gene demonstrated a specific melt peak at 81.5 °C. Following three months of treatment, a significant reduction of fourfold in VDRL titers was observed (1:16) (Table 2).

The complete ECDC typing system of the swab of lesion exudate was successfully obtained. The *arp* gene was amplified using the touchdown PCR method, and Sanger sequencing revealed the presence of 14 repeats of the 60 bp fragments on the *arp* gene. Additionally, the *tprEGJ* genes were identified as type E through RFLP analysis. The amplification products from the *tp0548* gene showed a homology of 100% with the G group. Altogether, these results established the genotype of *T. pallidum* isolate as 14 e/g. The 23 S rRNA gene of *T. pallidum* isolates carried the A2058G mutation, is associated with macrolide resistance. Nevertheless, the G1058C mutation in the 16 S rRNA gene was absent.

Discussion

In this study, we employed PCR and qPCR assays to detect *T. pallidum* in a genital lesion and whole blood sample from a patient with primary syphilis, as well as during the follow-up treatment. Overall, our results revealed that the lesion was positive by PCR, while the blood samples tested negative. This aligns with previous studies indicating that PCR is suitable for detecting *T. pallidum* in syphilitic lesions but less so in clinical blood samples [5]. The limited *T. pallidum* loads in blood samples during the primary stage seem to be a limiting factor for molecular assays. Yet, our study

demonstrated that qPCR was efficient in detecting treponemal DNA in whole blood samples, a sample type that typically contains lower *T. pallidum* loads compared to lesion samples.

Interestingly, we found that qPCR was unable to detect treponemal DNA in whole blood after one week of antibiotic therapy. This result suggests that qPCR, particularly when used with this sample type, may be valuable for monitoring infection progression and guiding appropriate treatments. Additionally, qPCR could be a promising approach to detecting low-copy *T. pallidum* DNA, especially during the early and latent stages, where current technics face challenges due to limited DNA copies in *T. pallidum* detection. For instance, a previous study using nested PCR identified *T. pallidum* DNA in 27% of biological samples, while qPCR detected it in 41% of them [12]. Our findings indicate that qPCR is a more sensitive, rapid, and accurate diagnostic method for syphilis in blood samples.

As discussed, nontreponemal antibody tests may present false-negative results, mainly due to the serofast state, which occurs in 1–2% of patients. Moreover, confirming treatment efficacy may be slow and problematic (taking up to 12 months) due to the non-specific nature of these tests [4]. Conclusively, syphilis management remains a challenge in clinical practice. Thus, the use of more sensitive and specific diagnostic methods is necessary for effectively managing syphilis. Our data also suggest that the standardized qPCR from lesion exudate and blood samples proves to be efficient in detecting *T. pallidum* DNA in clinical samples, making it a sensitive and reliable diagnostic tool for future studies on diagnosing and monitoring treatment in patients with various stages of syphilis.

Molecular methods play a crucial role in characterizing *T. pallidum* strains and contribute to our understanding of syphilis epidemiology. In Brazil, only a previous conference has described the circulation of three subtypes of *T. pallidum*: 14d/g, 14d/d, and 12b/d [13]. However, in our study, we identified a previously unreported subtype, 14 e/g, which had previously been reported in Australia [14], Denmark [15], Spain [16], and the Netherlands [17], not in Brazil. This finding highlights the dynamic nature of *T. pallidum* strains and emphasizes the importance of continuous monitoring to keep track of their distribution.

Reports on resistant *T. pallidum* strains in Brazil have been scarce and understudied. In a previous analysis, the resistance profile identified the A2058G mutation in the 23 S rRNA gene. Still, the A2059G mutation was not detected [13]. Our study further corroborates this trend, as the *T. pallidum* isolates in our patient also exhibited resistance to azithromycin due to the presence of the A2058G mutation in the 23 S rRNA gene, which is the most prevalent worldwide. On the other hand, we did not detect resistance to doxycycline. This is an encouraging result, as doxycycline remains an essential antibiotic for treating syphilis, especially in cases of azithromycin resistance. Nevertheless, continuous surveillance of resistance patterns is essential to promptly identify any emerging resistance to doxycycline or other treatment options.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42770-024-01342-w.

Acknowledgements This work was partially supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul (FUNDECT grants 092/2015 and 041/2017), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Universidade Federal da Grande Dourados (UFGD). JHFSQ received a scholarship from FUNDECT and S.S from CNPq. The funding agencies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical aspects This study was conducted with the approval of the Research Ethics Committee of the Universidade Federal da Grande Dourados under protocol number 1.402.529.

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Artigo 3: Diagnostic Microbiology & Infectious Disease (Qualis A4)

https://www.sciencedirect.com/journal/diagnostic-microbiology-and-infectious-disease/publish/guide-for-authors

Diagnostic Microbiology & Infectious Disease 109 (2024) 116333

Contents lists available at ScienceDirect

Diagnostic Microbiology & Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Molecular characterization of *Treponema pallidum* isolates from Brazil

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ARTICLE INFO

Keywords:
Treponemal DNA
polA gene
PCR
RT-PCR
ECDC scheme

ABSTRACT

Syphilis remains a public health concern in Brazil, and the data on the characterization and resistance of *Treponema pallidum* in Brazil is limited. The present study aimed to detect Treponema DNA in the lesions and blood samples obtained from individuals diagnosed with syphilis. The Brazilian isolates were submitted to the Enhanced Centers for Disease Control and Prevention (ECDC) scheme and also analyzed for resistance gene. Treponemal DNA from 18 lesions and 18 blood specimens were submitted for amplification using Polymerase Chain Reaction (PCR) and Polymerase Chain Reaction in Real Time (RT-PCR). Eight samples from lesions and eight from blood were positive in the RT-PCR analysis. Eight lesions and three blood samples were positive using PCR. Two samples exhibited azithromycin resistance. The Brazilian isolate types 14d/g, 14 d/c, 15d/c, and 15d/e were identified using the ECDC scheme. The three subtypes 14d/c, 15d/c, and 15d/e have been identified in Brazil for the first time.

1. Introduction

Syphilis is a curable sexually transmitted infection caused by the pathogen *Treponema pallidum* subsp. *pallidum*. The number of cases with the infection of this pathogen has increased significantly worldwide. In the last decade, the incidence of syphilis in Brazil has increased from 27,964 to 213,129 cases [1,2]. Syphilis screening usually involves serological assays for the detection of *T. pallidum*-specific and non-specific antibodies [3]. However, the delay between the onset of infection and the appearance of antibodies may lead to negative serological results in early-stage syphilis [4].

Screening tests are conducted to detect the antibodies to treponemal antigens for syphilis. However, these tests do not differentiate between active and past infections [5]. Therefore, it is necessary to develop novel screening tests with better detection accuracy. Polymerase Chain Reaction (PCR) assay also allows for the detection of *T. pallidum* in mucosal tissues and body fluids during the early stages of syphilis, even in the absence of lesions [6]. Polymerase Chain Reaction in Real Time (RT-PCR) is also highly effective in the early detection of syphilis lesions [7].

The molecular epidemiology of *T. pallidum* strains remains poorly understood despite the increase in the number of new cases of syphilis reported every year, particularly in South American nations such as Brazil. Molecular characterization is considered a valuable tool worldwide to understand the pathology of *T. pallidum* and identify its various strains. *T. pallidum* subtypes based on the analysis of arp (*tp0433*), tprE (*tp0313*), tprG (*tp0317*), and tprJ (*tp0621*) genes have been identified and documented [8]. In order to further improve the discrimination of *T. pallidum* strains, the *tp0548* gene has been incorporated into the typing method [9].

Penicillin has been used as the first-line antibiotic for syphilis worldwide. The other alternatives that are considered include ceftriaxone, doxycycline, and macrolides. However, macrolide resistance has been reported widely in *T. pallidum*, and studies have demonstrated that the prevalence of this resistance has been increasing over time [10,11]. Two specific mutations, A2058G and A2059G, in the 23SrRNA gene, are reportedly associated with macrolide resistance [12]. The present study aimed to detect treponemal DNA in the swab lesions and whole blood samples collected from patients diagnosed with early syphilis in Dourados, Brazil using PCR and RT-PCR assays. In addition, the Enhanced Centers for Disease Control and Prevention (ECDC) scheme was used in the present study to characterize the Brazilian *T. pallidum* isolates and evaluate the strains for macrolide resistance.

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https://doi.org/10.1016/j.diagmicrobio.2024.116333
Received 12 March 2024; Received in revised form 25 April 2024; Accepted 25 April 2024
Available online 27 April 2024

2. Material and methods

2.1. Study population

Between October 2018 and December 2022, all participants with suspected syphilitic lesions, who visited the Specialized Assistance Service in HIV/AIDS located in the municipality of Dourados in the Brazilian state of Mato Grosso do Sul, were requested to provide swab specimens for molecular detection and analysis. Each participant provided 3 mL of their peripheral venous blood, which was divided into two samples, one that was collected in the tube with the anticoagulant EDTA and the other collected in a dry tube, for molecular and serological assays, prior to treatment. All participants read and signed the informed consent form before being included in the study. This study was conducted with the approval of the Research Ethics Committee of the Universidade Federal da Grande Dourados under protocol number 1.402.529.

2.2. Syphilis serological tests

Three serological tests are conducted to diagnose syphilis as part of routine diagnostics in Brazil [13]. A point-of-care test (POCT) for syphilis (ABON Biopharm, Hangzhou, China) is conducted for the initial screening, which is followed by two confirmatory tests – the non-treponemal quantitative Venereal Disease Research Laboratory (VDRL) test (Abbott Murex, Dartford, UK) and the enzyme-linked immunosorbent assay (ELISA) that detects the anti-*T. pallidum* IgM and IgG antibodies in the samples (ICE Syphilis, DiaSorin, Saluggia, Italy). Accordingly, all serological tests were conducted in the present study in accordance with the manufacturers' specifications.

2.3. PCR detection of T. pallidum DNA

Genomic DNA was extracted from the lesion swab and whole blood samples of the study participants using the QIAamp DNA Blood Mini kit (Qiagen, Germantown, Maryland, USA) according to the manufacturer's protocol. The extracted genomic DNA was then quantified using the BioDrop DUO (BioDrop, Cambridge, UK). Next, the extracted DNA was evaluated for integrity and the presence of PCR inhibitors using the human *β-globin* gene. PCR assay targeting the *T. pallidum* polymerase I gene (*polA/tp0105*) was performed for the DNA extracted from the clinical specimens as described in a previous report [14]. The PCR reaction volume of 25 µL contained 2 µL of the extracted DNA, 1x PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.5 µM of each primer (Sense: 5'-TGCGCACGAAGATAGTGTGT-3'; Anti-sense: 5'-AGCAGACGTTACATCGAGCGGA-3'), and 1 U of Taq DNA Polymerase (Invitrogen, Brazil). The reaction was subjected to amplification in a T100 (Bio-Rad, Hercules, CA, USA) thermocycler with the following cycling conditions: initial denaturation at 94°C for 5 min; 40 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min; and followed by a final extension at 72°C for 5 min. For the positive and negative controls, pUC57/polA_d DNA [14] and nuclease-free water (Sigma-Aldrich, USA), respectively, were used.

2.4. Quantification of T. pallidum DNA using RT-PCR

The RT-PCR targeting the *polA* gene was performed using the SYBR Green JumpStart Taq Ready Mix (Sigma-Aldrich, MO, USA). The RT-PCR reaction mixture included 2 × SYBR Green Master Mix, 250 nM of each primer, and 2 µL of the extracted DNA, totaling a volume of 25 µL. The cycling conditions used were as follows: 94°C for 2 min, followed by 45 cycles of 94°C for 15 s, and 60°C for 1 min. A final melting step was also included, in which temperature was increased from 65°C to 95°C at a rate of 0.5°C/s. After amplification, the melting curve analysis was performed using the Bio-Rad CFX Manager software version 3.0.1224 (Bio-Rad Laboratories, Inc.).

2.5. Molecular typing and analysis of the antibiotic resistance gene

The ECDC typing of *T. pallidum* was determined by analyzing the genomic regions of the arp (*tp0433*) using touchdown PCR, as described in a previous report [15]. Nested PCR was performed to amplify the tprE (*tp0313*), tprG (*tp0317*), and tprJ (*tp0621*) genes using outer and internal primers, as described in a previous report [8]. The amplified nested PCR product was purified using a PureLink PCR Purification Kit (Invitrogen). After purification, the product was digested with *MseI* (Thermo Scientific). The digested product was subsequently analyzed through electrophoresis on a 2% agarose gel to identify the digestion pattern by comparing it with the published data [8]. The procedure for amplifying the *tp0548* gene was previously described [9].

The PCR assay to determine the resistance to azithromycin in *T. pallidum* was conducted based on the amplification of the 23S rRNA [15]. All PCR products were sequenced using Sanger sequencing (ABI 3730 xl DNA Analyzer, Applied Biosystems, USA). The sequencing data were analyzed using the BioEdit software (Version 7.2 available from https://bioedit.software.informer.com/7.2/).

3. Results

3.1. Study participants

A total of 23 participants were initially screened between October 2018 and December 2022. Five participants who were suspected of having syphilis and had failed to provide blood samples were excluded from the study. The remaining 18 participants who were diagnosed with early syphilis were enrolled in the study after testing positive in serological assays, such as the point-of-care test (POCT) for syphilis, VDRL, or ELISA. Among these 18 participants, 11 participants (61%) were diagnosed with primary syphilis based on the presence of a genital lesion. The remaining 7 participants (39%) had a skin or oral lesion and were, therefore, diagnosed with secondary syphilis (Fig. 1).

3.2. Detection of T. pallidum in clinical samples

A total of 36 clinical specimens (18 swab lesion samples and 18 whole blood samples) were assessed using PCR and RT-PCR analyses. Among these, 16 clinical specimens (8 swab lesion samples and 8 whole blood samples) presented positive results in the RT-PCR assay (Table 1), while 11 samples could be amplified in the PCR analysis. The participants with primary and secondary syphilis presented a higher frequency of positivity in the lesion samples compared to the whole blood samples. In the RT-PCR assay, 8 out of 18 whole blood samples were positive for *T. pallidum*, with 4 samples from participants in the primary stage and 4 from the secondary stage. In contrast, only 3 samples from participants in the secondary stage were positive in the PCR.

3.3. Molecular characterization of T. pallidum

Among the 8 strains positive for *T. pallidum* in lesion samples, 6 were successfully typed. Strains from 4 out of the 5 (80%) participants with primary syphilis were fully typed using the ECDC scheme. The subtypes 14d/g, 14d/c, 15d/c, and 15d/e were detected in strains from participants with genital lesion samples. In addition, two partial subtypes of Xd/x were detected (Table 1). Unfortunately, no typed strains from participants' whole blood samples were available. In regard to resistance to azithromycin in *T. pallidum*, 7 of the 16 samples that were positive in the *polA* gene RT-PCR analysis also tested positive for the 23S rRNA gene. Among these seven samples, three were successfully sequenced, and in two of these participants, the A2058G mutation was detected, while the remaining one participant did not have this mutation.

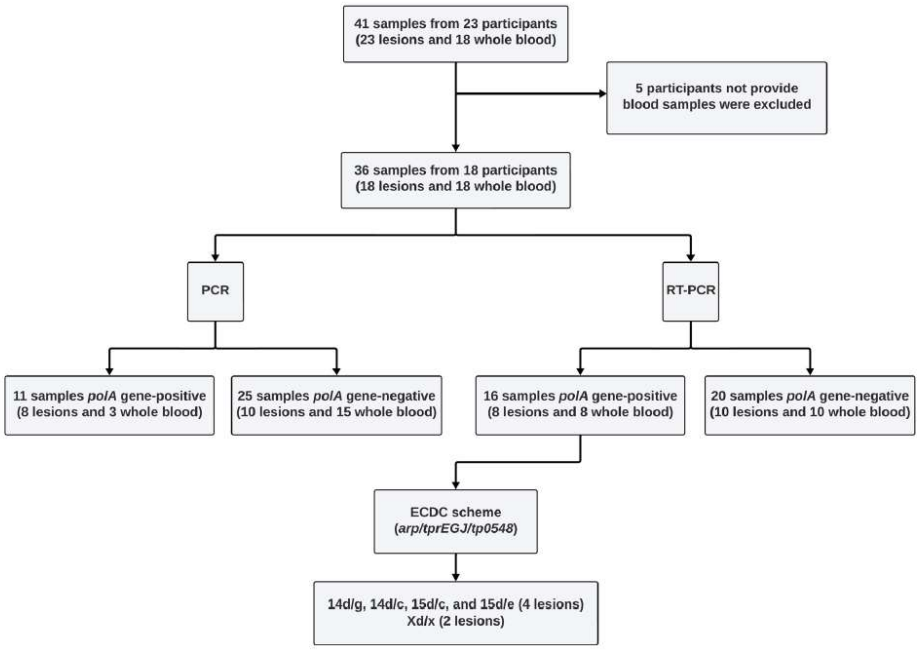

Fig. 1. Flow chart of the participants and clinical samples included in the ECDC scheme.

4. Discussion

PCR and RT-PCR analyses were conducted in the present study to detect *T. pallidum* in the lesions and whole blood samples from the participants diagnosed with primary and secondary syphilis. In addition, the *T. pallidum* strains currently prevalent in the Mato Grosso do Sul, Brazil were subjected to an investigation of their molecular properties using the ECDC scheme and determination of their resistance to azithromycin. The detection of *T. pallidum* DNA in patient blood samples during the primary stage of syphilis is challenging due to the typically low concentration of treponemal DNA in the samples. Nonetheless, the present study demonstrated that the RT-PCR method was effective in the detection of treponemal DNA in 36.4% (4/11) of the blood samples obtained from participants with primary syphilis, which typically have lower *T. pallidum* loads compared to the lesion samples [7]. It is noteworthy that previously, the RT-PCR method has been known for exhibiting reduced sensitivity in the case of blood samples from individuals with primary syphilis. For instance, previous studies using RT-PCR have reported *T. pallidum* DNA detection rates of 3% (2/35) for participants with primary syphilis and 21% (15/62) for participants with secondary syphilis [16,17]. The present study, therefore, highlighted the utility of RT-PCR in accurately diagnosing syphilis in blood samples with its superior sensitivity compared to the conventional PCR methods.

In Brazil, only three subtypes of *T. pallidum*: 14d/g (6), 14d/d (5), and 12b/d (1) were described in a conference summary [18]. In the present study, the 14d/g subtype was detected in just one participant. The 14 d/g subtype is the second most common worldwide, while 14d/f is the most prevalent and has been detected in 838 isolates across 18 countries between the years 2004 and 2018 [15,18–21]. In the present study, novel subtypes 14d/c, 15d/c, and 15d/e were identified in Brazil. Interestingly, the subtype 14d/c has been previously identified in clinical samples from participants in different countries, including South Africa (3) [21], China (3) [15], the Netherlands (2) [19], Spain (1) [20], Denmark (1) [20], and Japan (1)[20]. Moreover, this subtype was reportedly the predominant one in Zimbabwe, detected in 7 participants in a study [21]. The 15d/c subtype, on the other hand, has been reported in only a few nations, including South Africa (3) [21] and the Netherlands (2) [19]. The 15d/e type is rare and was identified only in the clinical samples from patients with neurosyphilis in the USA in 2009 [22] and primary syphilis in South Africa in 2011 [21]. Collectively, these findings demonstrate significant variation in the strains of *T. pallidum* across the world. However, it must be noted that the present study analyzed a limited number of isolates to assess the diversity of the *T. pallidum* isolates, and this should be considered when interpreting or generalizing the results. Additionally, a larger sample size could offer a more comprehensive understanding of the prevalence of azithromycin resistance in this population.

The present study has a few limitations. First, the recruitment of participants was interrupted for 20 months during the COVID-19 pandemic, introducing a significant delay in the study timeline and the small sample size, which could have affected the representativeness of the collected data. Second, studying *T. pallidum* presents inherent challenges as patients frequently delay seeking professional assistance upon the onset of syphilitic lesions, which leads to a potential underrepresentation in the sample set. Finally, although typing analyses provide valuable insights into the genetic diversity of *T. pallidum* strains, it is essential to consider the whole genome sequencing offers a more comprehensive approach, providing detailed genetic information that can uncover subtle variations among strains and potential associations with clinical outcomes. Despite this, our study was able to identify three novel subtypes of *T. pallidum* 14d/c, 15d/c, and 15d/e in Brazil, one of them is rare (15d/e type), with only two descriptions in the world.

The present study revealed that RT-PCR has great potential in detecting even low levels of treponemal DNA in clinical samples, which would be particularly beneficial in the early stages of infection. This is particularly relevant as the existing molecular techniques encounter challenges in detecting limited levels of *T. pallidum* DNA in blood

Table 1
Detection of Treponemal DNA in clinical samples from participants with early syphilis (n = 18) using PCR and RT-PCR assays.

Participants	Stage of syphilis	Specimen	PCR *polA*	qPCR *polA*	ECDC type	23S rRNA resistance mutations
1	Secondary	Skin lesion	+	+ Ct = 34.2	-	-
		Whole blood	+	+ Ct = 36.5	-	-
2	Primary	Genital lesion	+	+ Ct = 31.6	15d/e	S
		Whole blood	-	+ Ct = 34.2	-	-
3	Primary	Genital lesion	+	+ Ct = 33.5	14d/g	-
		Whole blood	-	+ Ct = 36	-	-
4	Secondary	Skin lesion	-	-		
		Whole blood	-	-		
5	Secondary	Skin lesion	-	-		
		Whole blood	+	+ Ct = 36.2	-	-
6	Secondary	Skin lesion	+	+ Ct = 31.3	-	-
		Whole blood	-	+ Ct = 35.6	-	-
7	Secondary	Skin lesion	-	-		
		Whole blood	-	-		
8	Primary	Genital lesion	+	+ Ct = 32	15d/c	R
		Whole blood	-	+ Ct = 36.7	-	-
9	Primary	Genital lesion	-	-		
		Whole blood	-	-		
10	Primary	Genital lesion	-	-		
		Whole blood	-	-		
11	Primary	Genital lesion	+	+ Ct = 31.2	14d/c	R
		Whole blood	-	+ Ct = 37	-	-
12	Primary	Genital lesion	-	-		
		Whole blood	-	-		
13	Primary	Genital lesion	-	-		
		Whole blood	-	-		
14	Primary	Genital lesion	-	-		
		Whole blood	-	-		
15	Primary	Genital lesion	+	+ Ct = 35.3	Xd/x	-
		Whole blood	-	-		
16	Primary	Genital lesion	-	-		
		Whole blood	-	-		
17	Secondary	Skin lesion	-	-		
		Whole blood	-	-		
18	Secondary	Oral lesion	+	+ Ct = 33.6	Xd/x	-
		Whole blood	+	+ Ct = 38	-	-

samples from primary and latent syphilis. In addition, although a limited number of samples were evaluated in the present study, *T. pallidum* diversity in Brazil and the resistance mutations commonly related to reduced susceptibility to therapy could be determined.

Funding sources

This work was partially supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico, Fundação de Apoio ao do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Universidade Federal da Grande Dourados (UFGD). JHFSQ and TSF received a scholarship from FUNDECT. BFL, EVOP and SS from CNPq. The funding agencies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

CRediT authorship contribution statement

Júlio Henrique Ferreira de Sá Queiroz: Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tiago da Silva Ferreira:** Resources, Investigation, Data curation. **Bruno Fernandes Lima:** Resources, Investigation, Data curation. **Emily Vitória de Oliveira Perez:** Resources, Investigation, Data curation. **Cindi Daniele de Oliveira Mello:** Resources, Investigation, Data curation. **Simone Simionatto:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.diagmicrobio.2024.116333.

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6 CONCLUSÕES

Os achados obtidos nesta tese têm demonstrado a importância de melhorias no diagnóstico da sífilis, tais como os métodos moleculares, evitando a transmissão da doença infecciosa nos estágios mais transmissíveis. Além disso, a sífilis não tratada gera danos irreversíveis para o paciente. Desta forma, um método de diagnóstico e o tratamento mais célere gera benefícios para toda a comunidade, ao encerrar o ciclo de transmissão dessa doença.

Investigamos *T. pallidum* detectados nas amostras clínicas de participantes com sífilis primária e secundária pelo método de tipagem molecular *ECDC*. O que possibilitou uma associação dos cinco subtipos de *T. pallidum* identificados neste estudo (14d/g, 14d/c, 14e/g, 15d/c e 15e/d) com os que estão em circulação pelo mundo. No entanto, mais estudos são necessários para ampliar o número de amostras clínicas avaliadas, e contribuir para o conhecimento dos possíveis mecanismos de infecciosidade e escape imunológico gerado por esses subtipos de *T. pallidum*.

O acompanhamento do tratamento da sífilis por meio de marcadores sorológicos torna-se demorado para uma queda significativa na titulação de anticorpos. O que acarretar dúvidas se o tratamento foi eficaz a curto prazo. Por outro lado, os métodos moleculares têm a capacidade de detectar *T. pallidum* em diversas amostras clínicas e estágios da sífilis. Desta forma, contribuir para melhoria do acompanhamento do tratamento da sífilis, principalmente, para gestantes que tem o alto risco de transmitir para o feto, sem o tratamento correto.

7 ANEXOS

PARECER DE APROVAÇÃO DO COMITÊ DE ÉTICA

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PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Epidemiologia e estudo genômico de cepas de Treponema pallidum
Pesquisador: Simone Simionatto
Área Temática:
Versão: 1
CAAE: 91700618.0.0000.5160
Instituição Proponente: FUNDACAO UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
Patrocinador Principal: Fundação Universidade Federal da Grande Dourados/UFGD-MS

DADOS DO PARECER

Número do Parecer: 2.756.689

Apresentação do Projeto:
A sífilis é uma infecção sexualmente transmissível (IST) causada pela bactéria Treponema pallidum, com elevada prevalência em vários países, incluindo o Brasil. A utilização de ferramentas de bioinformática e de biologia molecular para o estudo e identificação de genes e genomas constitui uma prática importante para o estudo de micro-organismos fastidiosos, como é o caso do T. pallidum. Baseando neste fato, esta pesquisa objetiva estudar a epidemiologia, o genoma, o perfil de resistência e a tipagem das cepas do T. pallidum. A associação dos dados epidemiológicos e moleculares irá contribuir para a compreensão da cadeia de disseminação da sífilis no Brasil. Trata-se de um estudo descritivo tipo inquérito transversal e de coorte que será realizado no período de Agosto de 2018 a Agosto de 2022. Serão incluídos no estudo pacientes com suspeita clínica para sífilis, de ambos os sexos e que desejarem participar mediante assinatura do Termo de Consentimento Livre e Esclarecido (TCLE) ou Termo de Assentimento Livre e Esclarecido (TALE). Estes pacientes que assinarem o termo serão submetidos a uma entrevista com base em um questionário estruturado para coleta de dados sócio demográficos, histórico de uso de drogas e álcool, histórico médico, histórico e presença de sinais e sintomas relacionados às doenças sexualmente transmissíveis. Após, será realizado a coleta de sangue e lesões para realizar os testes sorológicos e moleculares. O DNA treponêmico será sequenciado e as informações obtidas analisadas. As variáveis avaliadas serão inseridas em banco de dados do programa RedCap e analisadas utilizando-se programa estatístico SAS versão 9.1. Os dados dicotômicos ou

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categóricos serão analisados com o teste Qui-quadrado ou teste exato de Fisher. O sequenciamento do genoma do T. pallidum, a tipagem e determinação do perfil de resistência das cepas irão contribuir para estabelecer um padrão epidemiológico de disseminação deste micro-organismo no Brasil. Além disso, este estudo pretende auxiliar na implementação de novas políticas públicas de saúde, no que tange à promoção, prevenção e controle da sífilis.

Objetivo da Pesquisa:

Objetivo geral: Realizar o sequenciamento do genoma do T. pallidum, bem como a tipagem e perfil de resistência desta bactéria, buscando associar estes resultados aos dados epidemiológicos, contribuindo no entendimento da cadeia de disseminação da sífilis no Brasil.

Objetivos específicos:

- Coletar amostras de sangue e lesões de pacientes com suspeita clínica de sífilis;
- Realizar o diagnóstico sorológico da sífilis;
- Identificar fatores de risco associados à sífilis no Brasil;
- Realizar a tipagem e perfil de resistência das cepas do T. pallidum circulantes no Brasil; - Realizar o sequenciamento do genoma do T. pallidum;
- Conhecer a cadeia de disseminação da sífilis no Brasil e desta forma auxiliar os órgãos de saúde à compreender a magnitude dos problemas relacionados à esta IST;
- Contribuir com os órgãos de saúde no desenvolvimento de intervenções apropriadas e efetivas ao controle da sífilis;
- Promover e fortalecer as ações de Vigilância em Saúde no estado e país; - Formar Recursos Humanos para atuar na área da saúde.

Avaliação dos Riscos e Benefícios:

Os riscos restringem-se aos relacionados a algum tipo de desconforto durante a coleta de material biológico, onde os pacientes poderão sentir uma pequena sensação de desconforto no local da picada da agulha, sendo que poderá ser minimizado por ser coletado por profissionais devidamente treinados para a coleta de sangue e realizado com agulhas de pequeno calibre. Se houver algum mal estar, os pacientes receberão cuidados da equipe de saúde. Além disso, a entrevista poderá causar algum tipo de constrangimento, desconforto, estresses e cansaço ao responder o questionário. Os quais poderão ser minimizados sendo conduzidos em ambiente adequado e por profissionais treinados.

Como benefícios desta pesquisa, esperamos que a mesma contribua para o conhecimento da epidemiologia, genoma e perfil de resistência das cepas do T. pallidum circulantes no Brasil. A

associação dos dados genômicos e epidemiológicos irá auxiliar no entendimento da cadeia de disseminação da sífilis, visando apoiar intervenções com melhoria dos indicadores de saúde da população.

Comentários e Considerações sobre a Pesquisa:

O projeto apresenta objetivos claros, bem como o caráter voluntário da pesquisa, os critérios de inclusão e exclusão, e os riscos e benefícios da pesquisa.

Considerações sobre os Termos de apresentação obrigatória:

Considerando as questões de ordem ética necessárias para execução da pesquisa, o protocolo apresenta os termos obrigatórios, conforme previsto na RESOLUÇÃO No 466, DE 12 DE DEZEMBRO DE 2012. Entretanto, no TCLE e TALE não consta o campo para colocar o endereço e telefone do participante; explicitação que o (a) participante tem o direito de não responder as perguntas do questionário que ocasionem constrangimentos de qualquer natureza; campos para assinatura do (a) participante e do pesquisador nas duas páginas; numeração das páginas.

Recomendações:

Incluir no TCLE e TALE:

-Endereço e telefone do participante;

- Explicitação que o (a) participante tem o direito de não responder as perguntas do questionário que ocasionem constrangimentos de qualquer natureza;

- Colocar campo para assinatura do participante e pesquisador responsável nas duas páginas dos termos;

- Numerar as páginas. Exemplo: (página 01/02... página 02/02).

Conclusões ou Pendências e Lista de Inadequações:

Conclui-se pela APROVAÇÃO do presente protocolo de pesquisa, pois o projeto atende as exigências estabelecidas na RESOLUÇÃO No 466, DE 12 DE DEZEMBRO DE 2012.

Considerações Finais a critério do CEP:

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BASICAS_DO_PROJETO_1065142.pdf	18/06/2018 15:43:45		Aceito
Outros	declaracaoHUassinada.jpg	18/06/2018 15:41:44	Simone Simionatto	Aceito

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Continuação do Parecer: 2.756.689

Outros	TALE.pdf	14/06/2018 22:41:44	Simone Simionatto	Aceito
Orçamento	orcamento.pdf	14/06/2018 22:41:24	Simone Simionatto	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.pdf	14/06/2018 22:39:44	Simone Simionatto	Aceito
Outros	resolucaoFCBA.pdf	14/06/2018 22:22:51	Simone Simionatto	Aceito
Projeto Detalhado / Brochura Investigador	projetoTp.pdf	14/06/2018 16:22:04	Simone Simionatto	Aceito
Outros	smses.pdf	14/06/2018 14:23:07	Simone Simionatto	Aceito
Declaração de Pesquisadores	declaracaocris.pdf	14/06/2018 14:21:42	Simone Simionatto	Aceito
Declaração de Pesquisadores	declaracaocoordeandor.pdf	14/06/2018 14:21:21	Simone Simionatto	Aceito
Cronograma	Cronograma.pdf	14/06/2018 14:20:45	Simone Simionatto	Aceito
Folha de Rosto	folhaderostoA.pdf	14/06/2018 14:05:51	Simone Simionatto	Aceito
Declaração de Instituição e Infraestrutura	SCAN_20180614_105626857.jpg	14/06/2018 14:02:36	Simone Simionatto	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

DOURADOS, 05 de Julho de 2018

Assinado por:
Leonardo Ribeiro Martins
(Coordenador)

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