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AUTÓPSIA MINIMAMENTE INVASIVA (AMI) NO SERVIÇO DE VERIFICAÇÃO DE ÓBITOS DO CEARÁ: POSSIBILIDADES E AVANÇOS

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Tese apresentada ao Programa de Pós-graduação em Patologia da Faculdade de Medicina, da Universidade Federal do Ceará, como parte dos requisitos para obtenção do título de Doutor em Patologia.

Área de concentração: Patologia.

Orientador: Prof. Dr. Luciano Pamplona de Goes Cavalcanti

Coorientadora: Profa. Dra. Fernanda Montenegro de Carvalho Araújo

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A Deus,

À minha família.

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Não é sobre chegar no topo do mundo e saber que venceu, é sobre escalar e sentir que o caminho lhe fortaleceu. (VILELA, Trem-bala, 2017)

RESUMO

Introdução: a autópsia minimamente invasiva (AMI) configura-se como uma alternativa viável à autópsia convencional, considerada padrão-ouro para determinação das causas de morte, nas diversas situações em que não há possibilidade de abertura do corpo após a morte. As arboviroses permanecem um grave problema de saúde pública no Brasil, com aumento do número de óbitos a cada ano. Entretanto, há uma limitada capacidade de investigação desses óbitos, mesmo em locais com recursos técnicos para realização de autópsia como os serviços de verificação de óbito, em virtude da rejeição das famílias ao procedimento. **Objetivo**: Avaliar o potencial de uso da Autopsia Minimamente Invasiva (AMI) para vigilância de óbitos por doenças de notificação compulsória, com foco em arboviroses, no estado do Ceará. **Métodos:** uma parceria entre o Ministério da Saúde, as Universidades de São Paulo, Federal do Ceará e de Barcelona, da Secretaria de Saúde do Ceará e Centro Universitário Christus viabilizaram um curso teórico prático no Serviço de Verificação de Óbitos do Ceará onde foram treinados patologistas do serviço. Após esse treinamento foi possível propor, avaliar e validar um fluxo para implantação da AMI no SVO. **Resultados:** após o treinamento e revisão da literatura disponível mostramos a viabilidade da realização de AMI no SVO do Ceará. Um fluxo foi estabelecido e foram definidos critérios para a coleta, identificação, transporte e armazenamento das amostras segundo a metodologia de diagnóstico a ser utilizada. Em um período de três meses, 43 corpos foram submetidos a AMI no SVO. Destes, 21 (48,8%) foram encaminhados com hipótese diagnóstica de alguma arbovirose e, em sete (16,3%) o diagnóstico foi confirmado (seis de chikungunya; um de dengue); também foram confirmados casos de covid-19 (n = 9), tuberculose (n = 5), meningite (n = 4), criptococose (n = 1), doença de Creutzfeldt-Jakob (n = 1), neoplasia de mama (n = 1) e raiva humana (n = 1). Por fim, relatamos a confirmação de um óbito pediátrico por dengue grave que foi possível por conta da realização da AMI no SVO do Ceará. **Conclusão:** o fluxo criado e implantado permitiu a captação de óbitos suspeitos de arboviroses, além da confirmação de outras patologias de interesse da vigilância epidemiológica. A técnica parece ser promissora para ser utilizada nos casos em que a autopsia convencional não é permitida pela família.

Palavras-chave: Arboviroses; SVO; Autopsia Convencional; Autopsia Minimamente Invasiva.

ABSTRACT

Introduction: minimally invasive autopsy (MIA) is a viable alternative to conventional autopsy, considered the gold standard for determining the causes of death, in the various situations in which there is no possibility of opening the body after death. Arboviruses remain a serious public health problem in Brazil, with an increase in the number of deaths each year. However, there is limited capacity to investigate these deaths, even in places with technical resources to perform autopsies, such as death verification services, due to families' rejection of the procedure. **Objective**: To evaluate the potential use of Minimally Invasive Autopsy (AMI) for death surveillance in the state of Ceará. **Methods**: a partnership between the Ministry of Health, the Universities of São Paulo, the Federal University of Ceará and Barcelona, in addition to the Ceará Health Department and the Christus University Center, facilitated a practical theoretical course at the Ceará Death Verification Service where they were trained service pathologists. After this training, it was possible to propose, evaluate and validate a flow for implementing AMI in the SVO. **Results**: after training and reviewing the available literature, we showed the feasibility of performing AMI in the SVO in Ceará. A flow was established, and criteria were defined for the collection, identification, transport and storage of samples according to the diagnostic methodology to be used. Over a three-month period, 43 bodies underwent AMI at SVO. Of these, 21 (48.8%) were referred with a diagnostic hypothesis of an arbovirus and, in seven (16.3%) the diagnosis was confirmed (six of chikungunya; one of dengue); cases of covid-19 (n = 9), tuberculosis (n = 5), meningitis (n = 4), cryptococcosis (n = 1), Creutzfeldt-Jakob disease (n = 1), breast neoplasia (n = 1) were also confirmed. n = 1) and human rabies (n = 1). Finally, we report the confirmation of a pediatric death due to severe dengue that was possible due to the performance of AMI at the SVO in Ceará. **Conclusion**: the flow created and implemented allowed the capture of deaths suspected of arboviruses, in addition to the confirmation of other pathologies of interest to epidemiological surveillance. The technique appears to be promising for use in cases where conventional autopsy is not permitted by the family.

Keywords: Arboviruses; SVO; Conventional Autopsy; Minimally Invasive Autopsy.

	LISTA DE ABREVIATURAS E SIGLAS
AC	Autopsia Convencional
AgNS1	Antígeno Não Estrutural 1
AMI	Autópsia Minimamente Invasiva
AMI/US	Autópsia Minimamente Invasiva com base em ultrassom
AV	Autopsia Verbal
CHIKV	Vírus Chikungunya
DENV - 1	Virus Dengue sorotipo 1
DENV – 2	Virus Dengue sorotipo 2
DENV – 3	Virus Dengue sorotipo 3
DENV - 4	Virus Dengue sorotipo 4
FHD	Febre Hemorrágica da Dengue
FUNASA	Fundação Nacional de Saúde
HSJ	Hospital São José de Doenças Infecciosas
IEC	Instituto Evandro Chagas
MAYV	Virus Mayaro
MS	Ministério da Saúde
OMS	Organização Mundial da Saúde
OROV	Vírus Oropouche
RT-PCR	Reação de Cadeia de Polimerase via Transcriptase Reversa em Cadeia
SVO-RF	Serviço de Verificação de Óbito Dr. Rocha Furtado
ZIKV	Vírus Zika

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1 INTRODUÇÃO

1.1 Autópsia Convencional e Autópsia Minimamente Invasiva

Os primeiros relatos sobre autópsias remontam a muitos séculos. No passado, eram restritas aos anfiteatros de anatomia e, ao longo do tempo, passaram às instituições hospitalares e aos necrotérios públicos. O procedimento de autópsia tem valor inestimável na gestão da qualidade dos cuidados de saúde, no ensino e formação de profissionais da área da saúde, na colheita de tecidos para investigações e para diagnóstico da causa da morte que irão impactar nas estatísticas de mortalidade. Em situações específicas elas são obrigatórias. (VAN DEN TWEEL *et al.*, 2016).

Os avanços tecnológicos dos métodos de diagnóstico ocorridos nas últimas décadas não reduziram o valor da autópsia, e uma autópsia bem conduzida continua sendo a metodologia padrão-ouro no diagnóstico da causa da morte, além de ser importante na garantia da qualidade dos cuidados médicos prestados aos pacientes (GOLDMAN *et al.*, 1983; COSTACHE et al., 2014; BASSAT *et al.*, 2016).

O valor da Autópsia Convencional (AC) foi demonstrado em recentes epidemias como dengue, influenza A/H1N1, Zika, HIV e febre amarela (ARTEAGA *et al.*, 1998; PORTILLO *et al*, 2010; ARÉVALO *et al.*, 2010; CAVALCANTI *et al.*, 2015; SCHWARTZ *et al.*, 2017; DUARTE-NETO *et al.*, 2019). Seus resultados ajudaram a esclarecer não só a causa básica da morte, mas também a fisiopatologia das doenças. Mesmo após muitos anos, quando novas tecnologias se tornaram disponíveis, as análises de amostras de tecidos preservados em parafina revelaram novos conhecimentos sobre a pandemia de gripe espanhola de 1918 (SHENG *et al.*, 2011)

Contudo, apesar de revelar diagnósticos importantes, as autópsias têm diminuído nas últimas décadas, mesmo com o incentivo à continuidade de seu uso. (SHOJANIA *et al.*, 2003). Autópsia convencional (AC) com abertura de cavidades do corpo e exame das vísceras raramente é realizada em regiões com poucos recursos, devido a infraestrutura inadequada, falta de profissionais qualificados para conduzi-las e baixa aceitabilidade, dentre outras razões (COX *et al.*, 2011; OLUWASOLA *et al.*, 2009).

A Organização Mundial da Saúde (OMS) recomenda, como alternativa nesses contextos, o uso da Autopsia Verbal (AV) para investigação de todas as causas de mortalidade, principalmente as mortes ocorridas em domicílio (JOSH *et al.*, 2009; KAHN *et al.*, 2000). Sua

utilização não substitui a AC, mas é considerada como um complemento aos outros métodos de diagnóstico *post mortem* (BAILO *et al.*, 2022). A abordagem de AV teve grande utilidade devido a recomendação para não realização de AC na maior parte dos países, durante a pandemia da covid-19 (de SOUZA *et al.*, 2020).

Durante a pandemia de covid-19, alguns países decidiram não permitir AC, limitando a investigação adequada da fisiopatologia da doença e a confirmação de óbitos que não foram diagnosticados durante a evolução clínica (ARGUETA *et al.*, 2020). O uso da AV, complementada por exames laboratoriais específicos, tem mostrado bons resultados (de SOUZA *et al.*, 2020). Contudo, seu valor para definição da causa da morte tem sido questionado (BUTLER *et al.*, 2010). Embora forneça uma abordagem ampla, sua realização para diagnóstico etiológico ainda é limitada, pois pode classificar erroneamente algumas mortes causadas por doenças infecciosas (CASTILLO *et al.*, 2016).

Existem evidências do uso de procedimentos menos invasivos para obtenção de amostras *post mortem* a partir dos anos 1880 (WRIGHT Jr *et al.*, 2009). Em meados do século XX, algumas publicações de estudos envolvendo a utilização de agulhas, começaram a surgir em países de alta renda (TERRY *et al.*, 1955; WELLMANN *et al.*, 1969; FUROUDI *et al.*, 1995).

Nas últimas décadas, tem havido muitos investimentos em pesquisas relacionadas às técnicas minimamente invasivas para determinação da causa da morte com aumento importante no número de publicações (PAGANELLI *et al.*, 2020).

Diversos estudos foram realizados para validação da técnica, em diversos contextos, sobretudo em países de média e baixa renda (CASTILLO *et al.*, 2015, 2016; MARTÍNEZ *et al.*, 2016; MENENDEZ *et al.*, 2017; CASTILLO *et al.*, 2017; BASSAT *et al.*, 2017; HURTADO *et al*, 2018) revelando sua eficácia, aceitabilidade (BENSASI, K. *et al*., 2013; MAIXENCHS *et al,* 2016) e segurança (RAKISLOVA N. *et al*., 2021). Se realizada em conjunto por patologistas e radiologistas, a AMI tem sido uma alternativa sugerida à autópsia convencional em casos selecionados (THAYIL *et al.*, 2013).

Realizada no Sudeste do Brasil, durante a epidemia de febre amarela de 2018, a AMI guiada por ultrassom (AMI/US) mostrou-se eficaz tanto no diagnóstico da doença de base quanto na causa do óbito e teve 100% de concordância diagnóstica quando comparado com a AC. Assim, propôs-se que o método pode ser uma alternativa rápida e viável na determinação

do diagnóstico *post mortem* de febre hemorrágica viral causada por arbovírus (DUARTE-NETO et al., 2019).

1.1.1 Serviço de Verificação de Óbitos Dr Rocha Furtado

A Portaria ministerial 1405/2006 criou a Rede Nacional de Serviços de Verificação de Óbitos (SVO), com a finalidade de esclarecer a *causa mortis* em casos de morte natural, sem assistência médica, e os de causa mal definida, através da realização de necrópsia. Estabelece que os SVO que compõem a rede devem dar prioridade à investigação dos óbitos de interesse da vigilância epidemiológica, sobretudo os agravos de notificação compulsória, dentre eles, os óbitos suspeitos de arboviroses. A investigação de óbitos por arboviroses é obrigatória no Brasil (BRASIL, 2006).

No Ceará, o Serviço de Verificação de Óbito Dr. Rocha Furtado (SVO-RF), desde a sua fundação, em 2005, tem sido referência para realização de autópsias em óbitos suspeitos de arboviroses. Em parceria com o Laboratório Central de Saúde Pública (Lacen-CE), responsável, no estado, pela vigilância sorológica desde 1988 e pela vigilância virológica desde 1998, e o Núcleo de Vigilância Epidemiológica da Secretaria de Saúde do Estado do Ceará (NUVEP-CE); o SVO-RF contribuiu para a identificação, notificação e confirmação de óbitos suspeitos de dengue que não foram considerados pelos clínicos nos serviços de saúde (BRAGA, 2014; CAVALCANTI *et al.*, 2016, 2018).

Durante as epidemias de dengue, nos anos 2011 e 2012, e de chikungunya, em 2016 e 2017, o SVO-RF se deparou com dificuldades para realizar a identificação e confirmação dos óbitos, sobretudo em pessoas idosas, por recusa das famílias para autorização do encaminhamento do corpo ao serviço e realização da AC (CAVALCANTI *et al.*, 2019), um procedimento considerado desfigurante, demorado e desnecessário.

1.2 Arboviroses

As arboviroses (de arbovírus, abreviação para *arthropode-borne virus*) são infecções virais transmitidas a partir de um hospedeiro vertebrado e um artrópode hematófago (MUSSO; GUBLER, 2016). A maioria dos arbovírus pode ocasionar zoonoses que, geralmente, necessitam de um hospedeiro não-humano para preservação da espécie, sendo um motivo de grande problema de saúde pública mundial. As infecções geradas por essa classe de vírus manifestam um amplo espectro clínico, desde infecções assintomáticas, doença febril indiferenciada, doença hemorrágica, encefalites ou podendo levar a óbito (SILVA et al., 2023).

Os principais artrópodes transmissores desses vírus são os mosquitos e os carrapatos, que habitam principalmente regiões tropicais e temperadas. Os mecanismos mais eficazes para reduzir o risco de transmissão dessas arboviroses são: a eliminação de potenciais locais de reprodução de mosquitos e a aplicação de larvicidas em recipientes ou lugares que apresentem água parada. Foram relatadas outras formas de transmissão, tais como: de mãe para filho durante a gravidez, por transfusão sanguínea e por meio da relação sexual (MUSSO; GUBLER, 2016).

No Brasil, vários arbovírus têm sido identificados como importantes agentes causadores de infeções em humanos e compõem a lista nacional de doenças de notificação compulsória imediata. Os arbovírus de maior importância epidemiológica e de grande impacto para a saúde pública em ambientes urbanos no Brasil são: o vírus dengue (DENV), o vírus Zika (ZIKV) e o vírus chikungunya (CHIKV), transmitidos pelo mosquito *Aedes aegypti*.

Os primeiros casos de dengue relatados no Brasil datam do século XIX, quando foram descritos surtos em várias localidades. No início do século XX, Oswaldo Cruz iniciou a campanha brasileira de erradicação do vetor *A. aegypti* mas, somente na década de 40, com o apoio da Fundação Rockefeller, a campanha ganhou impulso e culminou com a erradicação do mosquito, até a década de 70 (FIGUEIREDO *et al.*, 1996; 2000).

O declínio no controle do mosquito vetor, associado à introdução de novos sorotipos virais na América Central, resultou na reintrodução dos DENV no Brasil após mais de cinquenta anos (FIGUEIREDO *et al.*, 2000). Em 1981, ocorreu um surto de dengue em Boa Vista, Roraima, onde foram isolados os DENV-1 e DENV-4 (OSANAI et al., 1983). Contudo, foi somente a partir de 1986 que a dengue se tornou um problema de saúde pública nacional, com a introdução do DENV-1 no município de Nova Iguaçu, no Rio de Janeiro (SCHATZMAYER et al., 1986). A partir daí a doença se expandiu causando surtos e epidemias por todo o país. Este cenário foi agravado ainda mais com a detecção do CHIKV, em 2014, e do ZIKV em 2015, que também passaram a causar surtos e epidemias no país (BRASIL, 2015).

A febre amarela, outra importante arbovirose que também pode ser transmitida em áreas urbanas pelo mosquito *A. aegypti*, é uma doença infecciosa febril aguda e de evolução abrupta. Atualmente tem seu ciclo de transmissão principalmente silvestre, por meio dos mosquitos dos gêneros *Haemagogus* e *Sabethes*. Sua primeira grande epidemia ocorreu em 1849-50 e desde 1942 não foram mais confirmados casos de transmissão urbana no Brasil, principalmente devido a inserção no calendário vacinal em regiões endêmicas. (BRASIL, 2022).

Outro arbovírus também encontrado no Brasil, porém com menor frequência e que acomete principalmente pessoas provenientes de florestas tropicais, o Mayaro (MAYV), tem como principal vetor o mosquito do gênero *Haemagogus*. Conhecida como a Febre de Mayaro, essa arbovirose vem sendo negligenciada por atingir populações de baixa renda. O primeiro surto documentado no Brasil foi descrito em 1955. Desde então, surtos e epidemias de febre de Mayaro foram registrados em 11 estados, principalmente no período de 2014 a 2016. Essa arbovirose apresenta-se com início súbito de febre, com quadro clínico, geralmente, de curso benigno (BRASIL, 2024).

Até o surgimento de CHIKV e ZIKV, o vírus Oropouche (OROV) era o arbovírus de maior incidência no Brasil depois do DENV, sendo transmitido principalmente pelos mosquitos *Culicoides paraensis* e *Culex quinquefasciatus*. Nos últimos 70 anos, foram relatados 30 surtos em humanos em países latino-americanos (Brasil, Peru, Colômbia, Guiana Francesa e Panamá) (WESSELMANN et al., 2024). O maior surto de OROV foi relatado no Brasil no final da década de 1970, com mais de 100.000 casos em humanos (SAKKAS et at., 2018). Porém, o número de genomas completos obtidos no Brasil ainda é escasso, o que dificulta a compreensão da evolução molecular, da dinâmica de transmissão e da carga viral do OROV (GUTIERREZ et at.,2020). Devido ao ressurgimento recorrente desse arbovirus na região amazônica e ao aumento notável na incidência e distribuição geográfica das infecções relatadas nos últimos anos, OROV vêm se tornando uma das ameaças arbovirais emergentes mais significativas na América Latina, fazendo-se cada vez mais necessário expandir o seu diagnóstico e sequenciamento (NAVECA et al., 2024).

Desde 2015, a tripla circulação simultânea do CHIKV, DENV e ZIKV tornou-se um grave problema de saúde pública, especialmente na região nordeste do País, onde são registrados muitos óbitos, impondo assim vários desafios aos serviços de saúde (CARVALHO & CAVALCANTI, 2016; FREITAS et al, 2017; BRITO *et al.*, 2016). A maioria dessas arboviroses apresenta epidemiologia, ciclos de transmissão em ambientes urbanos e início dos sinais clínicos muito semelhantes, o que pode acabar dificultando o diagnóstico. Não há tratamento específico para essas arboviroses. Entretanto, com um diagnóstico e manejo clínico apropriados, o índice de mortalidade pode ser reduzido. Assim, com a melhoria do diagnóstico laboratorial, bem como, um maior conhecimento da expressão clínica da doença por parte dos médicos tem permitido reconhecer suas consequências sobre vários órgãos e sistemas.

No Brasil, a investigação de óbitos suspeitos de arboviroses é obrigatória, visando identificar as causas e propor intervenções que evitem novos óbitos (BRASIL, 2016).

1.3 Arboviroses no Ceará

No Estado do Ceará, os primeiros relatos que sugerem a presença do vetor comum a dengue e a febre amarela, o *Aedes aegypti*, datam de 1851. Na ocasião aconteceu uma grande epidemia de febre amarela urbana, que resultou no óbito de mais de 900 pessoas (FRANCO, 1969).

Em função do sucesso da campanha de erradicação do *A. aegypti* promovida pela Fundação Rockfeller, responsável pelo combate ao mosquito no Brasil naquela época, o último caso de febre amarela no Ceará foi reportado em 1934. Contudo, com a detecção do *A. aegypti* em julho de 1984, anunciava-se sua reintrodução no Brasil. No Ceará, a identificação dos primeiros focos do mosquito ocorreu em Aquiraz, seguido de Fortaleza e Beberibe, em agosto do mesmo ano. Através de um levantamento realizado pela FUNASA, nos meses de janeiro a março de 1986, foram identificados, em Fortaleza, índices de infestação predial de até 75,93%. Foram identificados outros 65 municípios infestados pelo vetor na mesma época (LIMA *et al.*, 1985; ARAÚJO *et al.*, 2006).

1.3.1. Dengue no Ceará

O DENV é um Flavivírus de RNA de fita simples, com envelope lipídico e não segmentado, da família *Flaviviridae;* é o arbovírus mais prevalente e de rápida disseminação.

Dengue caracteriza-se por febre alta, dores musculares e articulares, além de outros sintomas que variam em gravidade. A doença envolve três fases: uma fase febril, fase crítica e fase de recuperação. A fase febril normalmente acontece por uma semana com sintomas como febre alta, sintomas gripais, cefaléia, vômitos, mialgia e dores articulares. A fase crítica, considerada de risco de vida, caracteriza-se por sintomas mais agudos, com vazamento de plasma e hemorragias. Na fase de recuperação ocorre melhora gradativa dos sintomas e recuperação da permeabilidade vascular (SIMMONS et al, 2012).

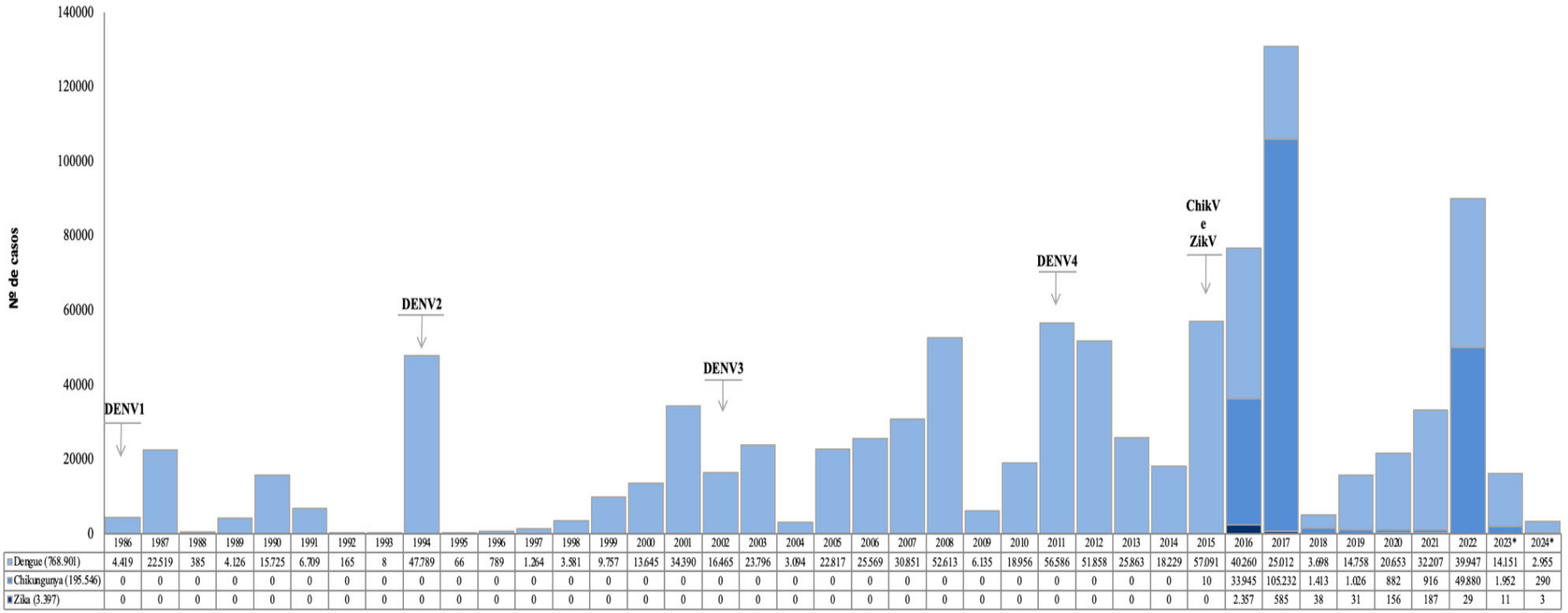
Dengue tem se manifestado de forma endêmica no Ceará, com os primeiros casos notificados em 1986, quando foi isolado o sorotipo DENV-1. Nas últimas décadas foram registradas, pelo menos, seis epidemias nos anos de 1987, 1994, 2001, 2008, 2011 e 2012 (CEARÁ, 2013; VASCONCELOS et al, 1998a). Um Boletim Epidemiológico de dengue de 2013 destaca as epidemias de 1994, pela confirmação dos primeiros casos hemorrágicos; a de 2008, pelo maior número de casos graves e atípicos, e 2011, com o maior número de casos clássicos confirmados (ARAÚJO *et al.*, 2012a; ARAÚJO *et al.*, 2012b; CEARÁ, 2013).

O sorotipo DENV-1 circulou isoladamente no estado até 1993, sendo reportados ao Ministério da Saúde mais de 50 mil casos, nenhum deles fatal. O início da circulação do sorotipo DENV-2 provocou uma grande epidemia que assolou o Estado no ano seguinte, com o aparecimento dos primeiros casos com manifestações hemorrágicas, predominando no município de Fortaleza. Foram notificados, nesse ano (1994), 178 casos suspeitos de febre hemorrágica da dengue (FHD). Apenas 26, dentre eles 11 óbitos, atenderam aos critérios da OMS e MS para confirmação do diagnóstico (VASCONCELOS et al, 1995).

Casos hemorrágicos começaram a ser notificados e continuaram a aparecer, destacando-se o ano de 2000, com letalidade de 75%, considerada a maior do Brasil (VILAR *et al.*, 2008). A circulação simultânea de três sorotipos, a partir de 2002, com a introdução do sorotipo DENV-3, aumentou o risco de evolução para casos hemorrágicos (ARAÚJO *et al.*, 2006). Vilar (2008) apontou um aumento do número de casos de FHD e uma redução significativa da faixa etária dos pacientes, provavelmente ocasionado pela circulação simultânea de três sorotipos virais por mais de duas décadas e a reemergência do DENV-2, após alguns anos sem circulação no Ceará. Observações semelhantes foram relatadas no Rio de Janeiro (TEIXEIRA *et al.*, 2009). A redução da idade média dos pacientes com evolução para formas graves, passou de 36,8 anos em 2001, para 30 anos em 2003 (CAVALCANTI *et al.*, 2011). A partir do final de 2011, com o isolamento pela primeira vez do sorotipo DENV-4 no Ceará, aumentou significativamente o risco de contrair a doença nos anos subsequentes (CEARÁ, 2014). O período de 2001 a 2012 foi marcado por epidemias com características sorológicas diferentes. O sorotipo DENV-2 predominando sobre o DENV-1, em 2001; o sorotipo DENV-3, co-circulando com o DENV-1, em 2006; circulação dos sorotipos DENV-2 e DENV-3, em 2008 e, em 2011 e 2012, os sorotipos DENV-1, DENV-3 e DENV-4 (OLIVEIRA *et al.*, 2014).

No ano de 2015, o Ceará vivenciou a maior epidemia de dengue em número de casos até então, com predomínio do sorotipo DENV-1 circulando com o DENV-3 e DENV-4. Houve confirmação de 57.091 casos distribuídos em 173 municípios cearenses e 72 óbitos. Desde então e, com a introdução do ZIKV e CHIKV, a série histórica apresenta circulação simultânea dos três arbovírus, como demonstra a Figura 1.

Figura 1. Série histórica das arboviroses dengue, Zika e chikungunya no Ceará, 1986 a 2024*.



	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023*	2024*
Dengue (768.901)	4.419	22.519	385	4.126	15.725	6.709	165	8	47.789	66	789	1.264	3.581	9.757	13.645	34.390	16.465	23.796	3.094	22.817	25.569	30.851	52.613	6.135	18.956	56.586	51.858	25.863	18.229	57.091	40.260	25.012	3.698	14.758	20.653	32.207	39.947	14.151	2.955
Chikungunya (195.546)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	33.945	105.232	1.413	1.026	882	916	49.880	1.952	290
Zika (3.397)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.357	585	38	31	156	187	29	11	3

Fonte: Boletim Epidemiológico das Arboviroses 2024, Secretaria de Saúde do Ceará.

A partir de abril de 2015, o Estado viveu um surto de uma doença caracterizada por febre baixa, erupção maculopapular, coceira, hiperemia conjuntival, dor e inchaço nos punhos e tornozelos, o que também acontecia em outros estados do Nordeste. O Hospital São José de Doenças Infecciosas (HSJ), onde foi implantada uma unidade sentinela com objetivo de esclarecer a etiologia da doença, colheu amostras de sangue de 40 pacientes na fase aguda da doença, enviadas ao Lacen-CE, para investigação etiológica (ARAÚJO *et al.*, 2016).

Alíquotas dessas amostras foram enviadas ao Instituto Evandro Chagas (IEC), no Pará, laboratório de referência nacional em arboviroses que conseguiu aprofundar as pesquisas sobre o Zika vírus (ZIKV). Das amostras testadas por RT-PCR, 14 obtiveram resultado detectável para o RNA do ZIKV; em nove amostras foi detectado DENV-1 por RT-PCR e em uma foi encontrado AgNS1. Assim, em maio de 2015, os primeiros casos de infecção por ZIKV foram confirmados laboratorialmente no Estado do Ceará (ARAÚJO et al, 2016b).

1.3.2. Zika no Ceará

O ZIKV pertence à família *Flaviviridae* e ao gênero *Flavivirus*, semelhante à classificação do DENV (GUBLER, 2007). Após a confirmação da circulação do vírus ZIKV em maio de 2015, o Ceará começou a registrar bebês, nascidos com suspeita de microcefalia, à partir do segundo semestre desse mesmo ano, com a confirmação de um natimorto com microcefalia, evidenciando a relação entre esta malformação congênita e a infecção pelo ZIKV na gestante. No final de 2015, o Ministro da Saúde do Brasil anunciou oficialmente a associação entre microcefalia em bebês e infecção pelo ZIKV Nos anos seguintes a doença demonstrou baixo número de registros no estado (ARAÚJO *et al.*, 2020).

Um estudo *post mortem* de recém-nascidos com infecção congênita por ZIKV no Ceará, Brasil, revelou microcefalia, ventriculomegalia, calcificação distrófica, depleção neuronal cortical grave em todos e artrogripose em seis, e uma coinfecção de ZIKV e DENV que foi evidenciada através de RT-PCR (SOUSA *et al.*, 2017). Nos últimos cinco anos não se detectou o ZIKV nas amostras processadas pelo Lacen-CE.

A prevalência de microcefalia aumentou de 0,06/1000 nascidos vivos em 2010 para 0,56 em 2015 e 3,22 em janeiro e fevereiro de 2016. Em junho de 2016 havia 417 casos suspeitos de microcefalia, com 317 confirmados (CAVALCANTI *et al.*, 2017).

A epidemia de ZIKV não pôde ser mensurada de forma adequada, no seu início, devido à falta de kits para realização do diagnóstico laboratorial, o que limitou a certeza do ano em que ele foi introduzido no Ceará. Mesmo assim, 2015, quando foram confirmados os primeiros casos, parece ter sido o ano da sua introdução (ARAÚJO et al., 2016).

1.3.3. Chikungunya no Ceará

A febre de Chikungunya é causada pelo CHIKV, um vírus RNA da família Togaviridae, gênero Alphavírus. A infecção pelo vírus, após o período de incubação, em média de 3 a 7 dias, pode evoluir em três fases: fase aguda ou febril, fase pós-aguda e fase crônica. A fase aguda é caracterizada por febre elevada, de início rápido com astenia intensa, poliartralgia, mialgia, cefaleia e erupção cutânea. Na fase pós-aguda, a febre desaparece, pode haver persistência ou agravamento da artralgia, incluindo poliartrite distal, exacerbação da dor articular nas regiões previamente acometidas na primeira fase e tenossinovite hipertrófica subaguda em punhos e tornozelos. Após essa fase, alguns pacientes podem evoluir para a fase crônica, com persistência dos sintomas, principalmente dor articular e musculoesquelética (BRASIL, 2017).

A taxa de casos assintomáticos é menor, e a percentagem de pacientes infectados que requerem atenção médica é maior do que na maioria das outras arboviroses. A idade avançada é o fator de risco mais óbvio associado à doença grave ou sintomas persistentes em adultos, enquanto nas populações pediátricas, os recém-nascidos têm um risco maior de agravamento da doença.

Os primeiros casos importados de chikungunya no Ceará foram confirmados em 2014. Em 2015, houve confirmação dos primeiros casos autóctones no Estado, nos municípios de São Gonçalo do Amarante, Fortaleza e Pires Ferreira. A partir de então, com a transmissão sustentada, o CHIKV causou uma grande epidemia em 2016 e 2017, levando alguns pacientes à fase crônica e outros à morte (CAVALCANTI *et al.*, 2019; SIMIÃO *et al.*, 2019). Nos anos seguintes, o cenário de chikungunya foi de baixa transmissão.

Em 2022, o CHIKV voltou a circular com potencial epidêmico, registrando transmissão elevada em todo o território do Estado. No período de 2016 a 2023 foram confirmados 191.608 casos e 280 óbitos (CEARÁ, 2024).

2 JUSTIFICATIVA

A investigação de óbitos por arboviroses é obrigatória no Brasil.

A investigação oportuna de todos os óbitos suspeitos ou confirmados de arboviroses tem sido uma premissa do SVO-RF, com intuito de estabelecer o diagnóstico, identificar outras condições relacionadas à morte, estabelecer padrões de lesão tecidual, avaliar efetividade das terapias e da imunização.

Contribuindo de forma efetiva para a detecção de óbitos por arboviroses não suspeitos pela clínica, durante a epidemia de dengue em 2011/2012, e de chikungunya em 2016/2017, no Ceará, o SVO se deparou com dificuldades para realizar a investigação através da autópsia, sobretudo por recusa das famílias em autorizar o encaminhamento do corpo e a realização da autópsia completa, sobretudo em pessoas idosas; um procedimento considerado desfigurante, demorado e desnecessário.

A autópsia minimamente invasiva - AMI é uma abordagem inovadora para obter amostras *post mortem* de órgãos-chave, cada vez mais reconhecida como uma metodologia robusta para investigação da causa da morte em países de média e baixa renda, validada para investigação de mortes por doenças infectocontagiosas.

Um fluxo simplificado de AMI, desenvolvido no SVO-RF, com boa aceitação pelos profissionais de saúde, agregando agilidade com risco reduzido, e presumivelmente, com melhor aceitação pela família, irá possibilitar o diagnóstico e a ampliação da investigação dos óbitos por arboviroses, por meio da punção por agulha com obtenção de amostras biológicas em ambiente hospitalar e/ou em locais que não disponham de infraestrutura e/ou pessoal qualificado para realização da autópsia completa.

3 OBJETIVOS

3.1 Objetivo geral

Avaliar o potencial de uso da Autopsia Minimamente Invasiva (AMI) para vigilância de óbitos por doenças de notificação compulsória, com foco em arboviroses, no estado do Ceará.

3.2 Objetivos específicos

- Estabelecer um fluxo para coleta de amostras biológicas utilizando a AMI para os casos em que a autopsia convencional não é possível.
- Ampliar o número de óbitos suspeitos de arbovírus investigados, melhorando a capacidade de diagnóstico da vigilância epidemiológica.
- Viabilizar a investigação de um maior número de óbitos, de interesse da vigilância epidemiológica.

4. MÉTODOS

Esta tese está baseada no Artigo 30 do Regimento Interno do Programa de Pós-graduação em Patologia da Universidade Federal do Ceará que, em seu parágrafo 4°, regulamenta o formato alternativo para Teses de Doutorado, e permite que a Tese possa ser apresentada em formato de artigo científico contendo anteriormente ao artigo, os elementos textuais: introdução e objetivos.

Este estudo seguiu os princípios éticos de pesquisa envolvendo seres humanos, conforme Resolução 466/12, do Conselho Nacional de Saúde, respeitando os princípios fundamentais de autonomia, beneficência, não maleficência, justiça e equidade. Este trabalho foi aprovado pelo CEP do Centro Universitário Christus, por meio do CAAE: 27162619.1.0000.5049, número do Parecer: 3.851.684 (anexo A).

Assim sendo, esta TESE é composta de duas partes. Uma textual contendo introdução, justificativa e objetivos, além de 3 capítulos, conforme detalhados abaixo:

4.1. Capítulo 1 (A1) (https://doi.org/10.1371/journal.pntd.0009629).

Use of minimally invasive autopsy during the COVID-19 pandemic and its possibilities in the context of developing countries. ***Plos Neglected Tropical Diseases***

Autores: **Deborah Nunes Melo**, Tânia Mara Silva Coelho, Giovanna R. P. Lima, Carolina Gomes Fernandes, Bruno Cavalcante Fales de Brito Alves, Fernanda Montenegro de Carvalho Araújo, Renata Aparecida de Almeida Monteiro, Jaume Ordi, Paulo Hilario do Nascimento Saldiva, Luciano Pamplona de Góes Cavalcanti

4.2. Capítulo 2 (A3) (https://doi 10.1590/S2237-96222024v33e2024008).

Usefulness of minimally invasive autopsy in the diagnosis of arboviruses to increase the sensitivity of the Epidemiological Surveillance System in Brazil: Experience from Ceará. ***Epidemiologia e Serviços de Saúde***.

Autores: Lívia Mendes de Almeida, **Deborah Nunes Melo**, Manuella Mendonça da Silva, Pedro Mansueto Melo de Souza, Fernanda Kézia de Sousa Silva, Tânia Mara Silva Coelho, Shirlene Telmos Silva de Lima, Anacelia Gomes de Matos Mota, Renata A A Monteiro, Paulo H N Saldiva, Geraldo Gileno de Sá´ Oliveira, Luciano

4.3. Capítulo 3 (https://doi.org/10.3390/tropicalmed7070123).

Post-Mortem Diagnosis of Pediatric Dengue Using Minimally Invasive Autopsy during the COVID-19 Pandemic in Brazil. ***Tropical Medicine and Infectious Diseases*** (Fator de impacto: 2.9)

Autores: **Deborah Nunes Melo**, Giovanna R. P. Lima, Carolina G. Fernandes, André C. Teixeira, Joel B. Filho, Fernanda M. C. Araújo, Lia C. Araújo, André M. Siqueira, Luís A. B. G. Farias, Renata A. A. Monteiro, Jaume Ordi, Miguel J. Martinez, Paulo H. N. Saldiva, Luciano P. G. Cavalcanti.

5. RESULTADOS

5.1. Capítulo 1 (artigo publicado)

Use of minimally invasive autopsy during the COVID-19 pandemic and its possibilities in the context of developing countries.

Plos Neglected Tropical Diseases

PLOS NEGLECTED TROPICAL DISEASES

VIEWPOINTS

Use of minimally invasive autopsy during the COVID-19 pandemic and its possibilities in the context of developing countries

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Coronavirus Disease 2019 (COVID-19) cases have spread quickly across the globe, but the deaths are distributed unevenly. These differences are related to several reasons, including the lack of access to diagnosis and socioeconomic status [1–3].

In this scenario, despite the advances in diagnostic methods in recent decades, a well-performed autopsy remains the gold standard methodology for diagnosing the cause of death [4].

The value of the autopsy has been shown in recent epidemics such as dengue, influenza A/H1N1, Zika, HIV, and yellow fever [5–9]. The autopsy findings helped to clarify not only the basic cause of death, but also the pathophysiology of the disease. These discoveries were possible even after many years when new technologies became available, as exemplified by the studies on paraffin-preserved material from the Spanish flu pandemic of 1918 [10].

Also, autopsies make an indispensable contribution to medical education and training. They provide a unique situation to observe systemic manifestations of different diseases, providing the basis for a medical training.

However, the number of autopsies performed globally is decreasing progressively. This phenomenon is partly due to the lack of limited resources and technical challenges, especially in developing countries. Cultural barriers and the reluctance of families to provide informed consent are additional factors contributing to the decline in autopsy numbers.

Moreover, due to the COVID-19 pandemic, some countries have decided not to allow complete autopsies, which limits the proper investigation of the disease pathophysiology and the death confirmations that were not diagnosed during clinical evolution [11]. In this scenario, some countries are expanding the use of verbal autopsy complemented by specific laboratory tests with good results [12]. However, although verbal autopsy provides a broad approach, its performance for etiological diagnosis is still limited, as it may misclassify some deaths caused by infectious diseases [13].

Thus, the minimally invasive autopsy (MIA) has emerged as an innovative strategy. It is a simple systematic collection technique that utilizes tissue samples from various organs and

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body fluids, configuring a fast, nondisfiguring procedure, with easy technical applicability that can provide robust data for surveillance, especially in regions with limited resources, being useful to guide strategies for prevention, control, and treatment of diseases [4,14–16].

In addition to health professionals considering it more acceptable, this technique stands out for overcoming the low acceptability and feasibility of a complete autopsy in some regions, which contributes to reducing the stigma of more invasive current practices [4,17].

In the study carried out in Southeastern Brazil during the yellow fever epidemic of 2018, the ultrasound-guided MIA (MIA/US) proved to be effective in both diagnosing the underlying disease and the cause of death and had a 100% diagnostic agreement when compared with conventional autopsy [9]. Similar findings were recently reported in Barcelona with 6 deaths by COVID-19, but without the use of an image resource [16].

The application of imaging techniques may create the impression that MIAs are expensive. However, the adaptation of methodologies and adequate personnel training proved cost-effective diagnostic tools in less developed regions [18,19].

Specifically, in the case of COVID-19 pandemic, in addition to being efficient in establishing the diagnosis of infections, it is a method that offers more safety to the professionals and can be performed with the body closed and surrounded by a plastic cover.

In 2020, the first autopsies using MIA technique were promoted in COVID-19 victims by the project Plataforma de Imagens na Sala de Autópsia (PISA), in the city of São Paulo. A year later, aiming to clarify the role of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) in lung and systemic injures, MIA was started in the state of Ceará, at the Serviço de Verificação de Óbitos Dr Rocha Furtado [20,21].

The wide acceptance of MIA in combination with simple imaging methods such as ultrasound scan offers a readily available, affordable, and safe postmortem diagnostic technique. Implementing MIA in developing countries can increase the accuracy of epidemiological surveillance indicators and overcome several barriers that prevent the performance of complete autopsies.

This tool can play an important role in improving the surveillance of causes of death in locations where infectious diseases are a common cause of mortality. The application of MIA should not eliminate the need of reinforcing death verification services or performing public verbal autopsies, even though it demonstrates limitations at the individual level, but which are still useful in low-budget situations. It is necessary to increase access to this technique, making it possible to expand the knowledge about the pathophysiology of these emerging diseases that lead thousands of people to death. In addition, its use can expand the range of pathologies that can be seen in biopsies, in the detection of emerging diseases, and even in the diagnosis of chronic diseases.

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5.2. Capítulo 2 (artigo publicado)

Usefulness of minimally invasive autopsy in the diagnosis of arboviruses to increase the sensitivity of the Epidemiological Surveillance System in Brazil: Experience from Ceará.

Epidemiologia e Serviços de Saúde.

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EXPERIENCE REPORT

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Usefulness of minimally invasive autopsy in the diagnosis of arboviruses to increase the sensitivity of the Epidemiological Surveillance System in Ceará, Brazil

Utilidade da autópsia minimamente invasiva no diagnóstico das arboviroses para ampliação da sensibilidade do Sistema de Vigilância Epidemiológica no Brasil: experiência do Ceará

Utilidad de la autopsia mínimamente invasiva en el diagnóstico de arbovirus para aumentar la sensibilidad del Sistema de Vigilancia Epidemiológica en Brasil: experiencia de Ceará, Brasil

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ABSTRACT

Objective: To create a protocol for performing minimally invasive autopsies (MIA) in detecting deaths from arboviruses and report preliminary data from its application in Ceará state, Brazil. **Methods:** Training was provided to medical pathologists on MIA. **Results:** A protocol was established for performing MIA, defining criteria for sample collection, storage methods, and diagnoses to be carried out according to the type of biological sample; 43 MIAs were performed in three months. Of these, 21 (48.8%) arrived at the Death Verification Service (SVO) with arboviruses as a diagnostic hypothesis, and seven (16.3%) were confirmed (six chikungunya cases and one dengue case); cases of COVID-19 (n = 9), tuberculosis (n = 5), meningitis (n = 4), cryptococcosis (n = 1), Creutzfeldt-Jakob disease (n = 1), breast cancer (n = 1), and human rabies (n = 1) were also confirmed. **Conclusion:** The protocol implemented enabled identification of a larger number of suspected arbovirus-related deaths, as well as confirmation of other diseases of interest for surveillance.

Keywords: Arbovirus Infections, Autopsy, Epidemiologic Surveillance Services, Investigative Techniques.

INTRODUCTION

Concerned with the arbovirus scenario in Brazil,[1-3] the country's Health Ministry recommends that deaths suspected to be due to this condition be investigated.[4] Determining the cause of these deaths continues to be a challenge.[5] Autopsy undoubtedly contributes to improving understanding of how microorganisms cause diseases, especially emerging and re-emerging diseases.[6]

Autopsies performed by the Death Verification Service (*Serviço de Verificação de Óbito* - SVO) of the Ceará State Health Department contributed to greater detection of deaths due to dengue, being one of the largest sources of notification of suspected deaths due to dengue in 2011-2012 and chikungunya in 2016-2017 in its territory.[7-10]

However, refusal to authorize autopsies is considerable, due to lack of information, prejudice or pressure from burial services.[11,12] Therefore, there is greater need to use safer and less invasive techniques to obtain organ samples for post-mortem analysis, potentially more acceptable to relatives or guardians of those who died.[13] Another aspect to be mentioned about the issue was the emergence of the COVID-19 pandemic, which led most services to suspend autopsies for safety reasons.[14-16]

The objective of autopsies is to obtain more information about the pathological processes and determine the factors contributing to death.[17] If, on the one hand, this practice has become increasingly difficult in cases in which the family does not allow it to be performed,[11] on the other hand minimally invasive autopsy (MIA) has become an increasingly used method for collecting samples from key post-mortem organs.[12,17-19] MIA is a relatively simple technique for collecting tissue samples from various organs and body fluids; a quick, non-disfiguring procedure, easy to perform and capable of providing robust data for health surveillance.[20-23]

Study contributions	
Main results	A protocol was developed to perform minimally invasive autopsies (MIAs) in Death Verification Services (SVO), capable of expanding the system's capacity to identify a greater number of deaths suspected to be due to arboviruses.
Implications for services	The experience suggests that in-service trained health professionals are able to perform MIA, and that use of this technique in SVOs has been shown to be capable of increasing the system's sensitivity in detecting deaths of interest to public health.
Perspectives	Trained professionals will be able to collect biological material in hospitals, through MIA, in cases of interest for health surveillance and when family members do not allow a complete conventional autopsy to be performed.

The objective of this study was to establish a protocol for performing MIAs in detecting deaths due to arboviruses, and to report preliminary data on its implementation in the state of Ceará, Brazil.

METHODS

This was a report of an experience developed in partnership between the *Universidade Federal do Ceará* Pathology and Public Health Postgraduate Programs, the Ceará State Health Department, the Dr. Rocha Furtado Death Verification Service (SVO), the Faculty of Medicine of the *Centro Universitário Christus* and the Ceará Central Public Health Laboratory (*Laboratório Central de Saúde Pública* - LACEN/CE), as well as the *Hospital Clínic de Barcelona* Department of Anatomic Pathology and Microbiology and the *Instituto*

de Salud Global de Barcelona (ISGlobal), Spain, to implement the MIA technique in Ceará.

The project subsequently received funding from the Brazilian Health Ministry, which, among other items, paid for the team of pathologists from Spain to come to Brazil to provide in-service training at the Dr. Rocha Furtado SVO. However, the onset of the COVID-19 pandemic led to the closure of Fortaleza's international airport; subsequently, autopsies were suspended in accordance with Ministry of Health guidelines.[17] At that time of pandemic crisis, the service's need to implement MIA increased, given the growing number of corpses arriving at the SVO without an established underlying cause, whereby several of those deaths occurred at home.

Given the urgent need to start using MIA, the Health Ministry facilitated negotiations between the Ceará group and the *Universidade de São Paulo* team to enable some Ceará pathologists to be trained. As part of this partnership, three professionals were sent to be trained in São Paulo (Figures 1A and 1B). The first experimental MIA in Ceará was performed in January 2021.[24] As such the Ceará SVO was the second such service in Brazil to put this technique into practice (Figures 1C, 1D, 1E and 1F).

As the pandemic subsided, it was possible to carry out training at the Ceará SVO, between November 7th and 11th, 2022, with the participation of and experience sharing between professionals from São Paulo, Barcelona/Spain, and the Health Ministry. Nine pathologists from Ceará received in-service MIA training. Once the local team had been trained, it was possible to establish work flows within the SVO and begin performing MIAs.

While the professionals were still considered to be in the training period, in those cases in which the family granted authorization, MIA was performed, followed by autopsy, with the aim of comparing the imaging findings with those of the biological samples sent to the reference laboratory. In those cases in which biological material was collected using both techniques, the procedures were performed by different pathologists. The result as to agreement between the two procedures occurred directly, by comparing the target organ macroscopic and microscopic findings.

The study project was approved by the *Centro Universitário Christus* Research Ethics Committee on February 20th 2020, as per Certificate of Submission for Ethical Appraisal No. 27162619.1.0000.5049 and Opinion No. 3.851.684.

RESULTS

During the first three months after in-service training, 43 MIAs were performed, of which 21 (48.8%) cases arrived at the SVO with arboviruses as a diagnostic hypothesis, while the remaining 22 (52.2%), were suspected cases of other conditions. Among the 21 deaths suspected to be due to arboviruses, seven (16.3%) were laboratory confirmed: six due to chikungunya and one due to dengue. The female sex predominated (79.2%) and average age was 54 years (< 1 to 100), with emphasis on the elderly (39% aged 70 or over).

Among the other conditions investigated, there were nine confirmed cases of COVID-19, five tuberculosis, four meningitis, one cryptococcosis, one Creutzfeldt-Jakob disease, one breast neoplasm and one human rabies case.

Both techniques (MIA and conventional autopsy) were performed in 30/43 (60.7%) of the cases. The samples sent to the LACEN/CE laboratory had IgM and RT-qPCR positive results, both in blood, cerebrospinal fluid and viscera (brain and spleen) samples. Percentage agreement between the findings using the two techniques was greater for the brain, heart, lung and liver, and more complex for the spleen. It was possible to clearly identify findings such as liver hepatocyte disarrangement, acute kidney tubular necrosis, in addition to pulmonary alveolar edema and hemorrhage (Figures 2 and 3).

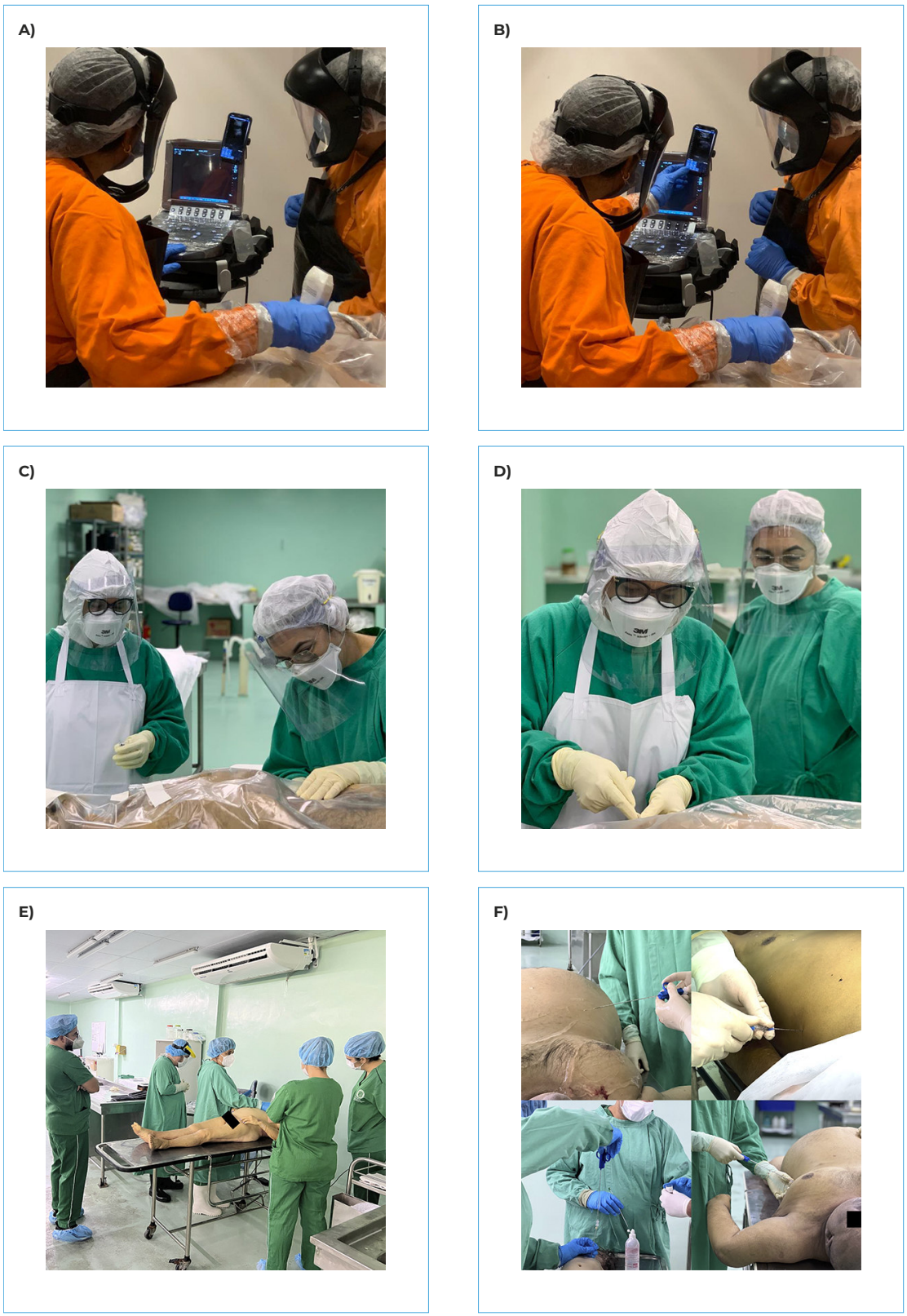
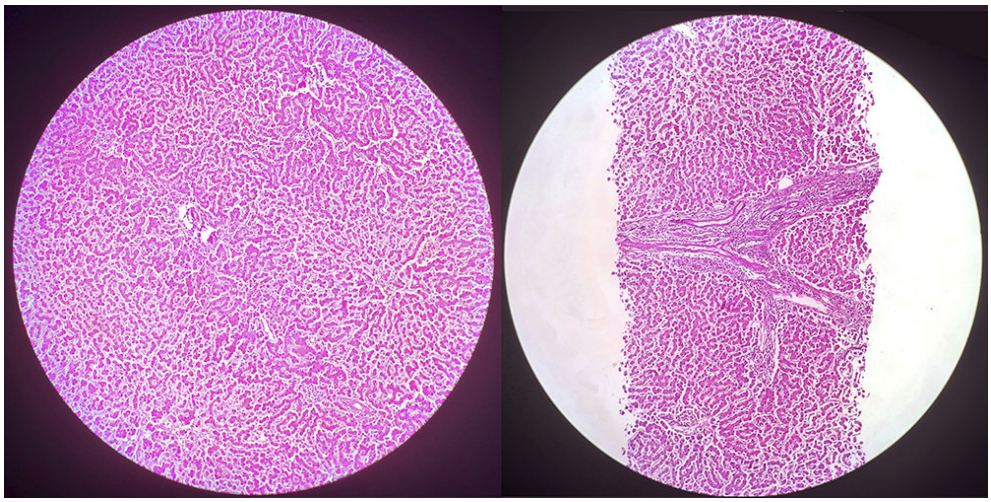


Figure 1 – (A) training conducted on the Image Platform, *Universidade de São Paulo* Autopsy Room; (B) training conducted on the Image Platform, *Universidade de São Paulo* Autopsy Room; (C) performance of minimally invasive autopsy in Ceará; (D) performance of minimally invasive autopsy in Ceará; (E) performance of minimally invasive autopsy in Ceará; (F) performance of minimally invasive autopsy in Ceará

1)



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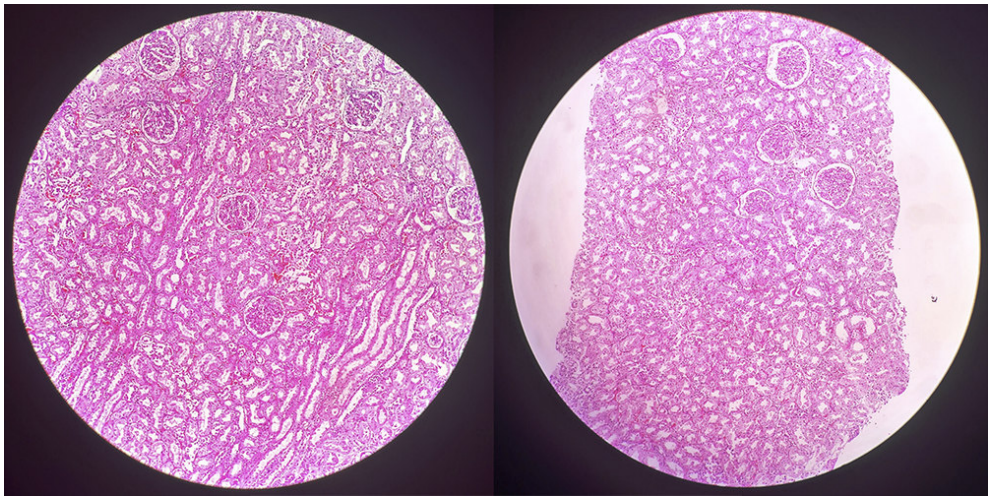

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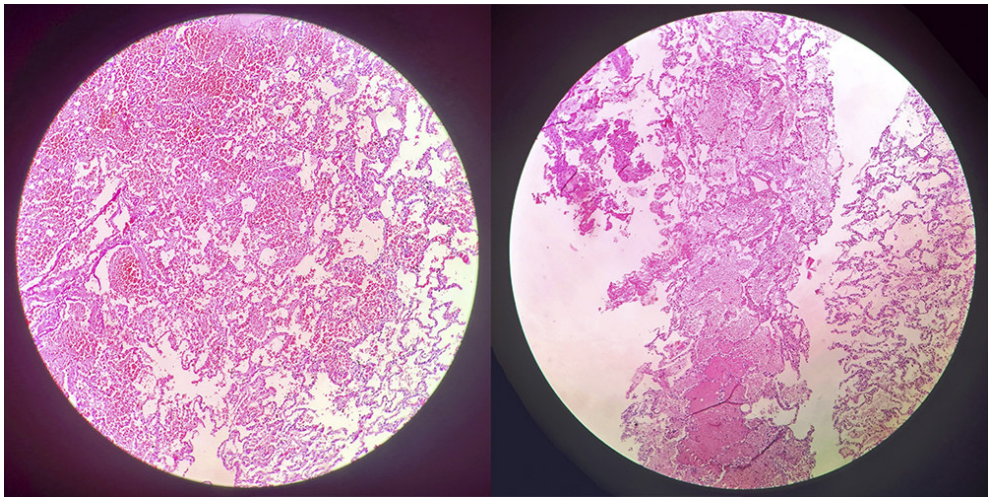

Figure 2 – Comparison of histological sections of samples collected via autopsy (on the left) and via minimally invasive autopy (on the right) (Hematoxylin-Eosin, 100x)

Legend: 1) Hepatocyte disarrangement in liver samples; 2) Acute tubular necrosis in kidney samples; 3) Alveolar edema and hemorrhage in lung samples.

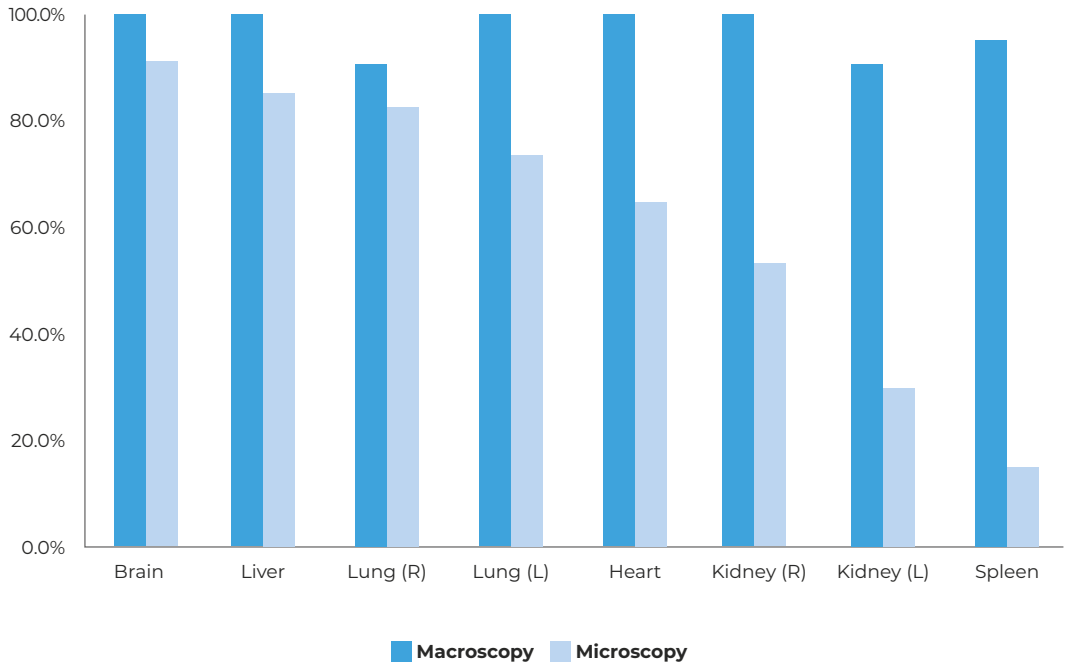

Figure 3 – Percentage agreement between tissue collected via conventional autopsy and punctures via minimally invasive autopsies performed on the first 30 deaths investigated at the Ceará Death Verification Service (*Serviço de Verificação de Óbito* - SVO), January 29 – May 7, 2023

Notes: Macroscopy = when the pathologist was able to collect fragments that, macroscopically, appeared to be from the puncture target organ; Microscopy = when it was confirmed, microscopically, that the fragment collected via puncture corresponded to the puncture target organ.

Based on this initial experience, a work flow was created and the necessary routines for carrying out MIA at the SVO were established, in addition to defining the following selection criteria:

a) death referred to the SVO with arbovirus as a diagnostic hypothesis; or
b) death referred to the SVO whereby suspicion of arbovirus is raised following interview with family members; or
c) death for which family members or guardians do not authorize conventional autopsy; or
d) death due to high-risk disease, whereby autopsy is contraindicated.

If MIA was indicated by any of these criteria, family member authorization was requested for performing minimally invasive autopsy, upon completion of an Informed Consent Form regarding the procedures involved. Once such authorization was received, the post-mortem study was begun using MIA, separating and identifying all the material to be used. Semi-automatic coaxial needles, of the TRU-CUT type, 16G x 20 cm long for adults and 14G x 9 cm long for children were used to perform MIA. Individual needles were used for each organ biopsied: brain, heart, right lung, left lung, liver, spleen, right and left kidneys, in that order.

The fragments collected using MIA were distributed for biomolecular, immunohistochemical and histopathological analyses, according to the following protocol:

– eight needles, with their respective eight guide wires, for puncturing the eight key organs (brain, heart, right lung, left lung, liver, spleen, right kidney and left kidney);

– ten formaldehyde-free cryotubes, for fresh packaging of blood, cerebrospinal fluid and eight key organ samples, to be sent for serological and molecular biology analysis at the LACEN/CE laboratory;

– eight cryotubes containing 10% buffered formaldehyde, for packaging the fragments of each of the eight key organs, to be sent for immunohistochemical analysis at the LACEN/CE outsourced reference laboratory;

– eight cryotubes containing 10% buffered formaldehyde, for packaging the fragments of each of the eight key organs, to be processed for histopathological analysis at the SVO itself.

After performing all MIA punctures, the corpses were transferred from the stretcher to the necropsy table and underwent autopsy, involving opening the cranial and thoracoabdominal cavities. Fragments from all key organs were also collected and each of them were fully examined, choosing the best sample from each organ for analysis.

Once MIA and the conventional autopsy had been completed, all samples were sent to the laboratories and their results were later compared in order to validate the technique regarding arbovirus cases.

DISCUSSION

The creation and implementation of the protocol by the Ceará SVO enabled cases of deaths of interest to epidemiological surveillance in which the corpses did not have family authorization for performing a conventional autopsy to undergo the MIA technique, enabling the collection of biological material to investigate the cause of death. Use of MIA made it possible to increase both the number of suspect deaths to be investigated and also the sensitivity of the investigation system.

One limitation of the study that stands out is that, even though it is a safe, fast, accessible technique with greater acceptability, regardless of religious and ethical impediments,[25,26] use of MIA met with resistance from some pathologists. It is also worth mentioning the difficulty in identifying and puncturing small focal lesions, which affect only a small portion of a given organ. Focal lesions, such as nodules or abscesses, for example, may not be punctured if random punctures are performed. This limitation is partially resolved with the use of ultrasound, which enables identification of a large number of focal lesions, as well as enabling puncturing to be directed towards them. A further limitation relates to identifying focal lesions that do not show changes when using ultrasound, such as in areas of myocardial ischemia. For this type of lesion, however, there are no MIA techniques that satisfactorily replace the macroscopic analysis that conventional autopsy provides. Despite these limitations, it is worth highlighting that using MIA it is possible to identify the etiological agent in the majority of deaths due to infectious causes.[21]

Considering the primary objective of the protocol, i.e. to demonstrate the usefulness of MIA in diagnosing arboviruses, and given that it is known that arbovirus infections are systemic and affect organs in a diffuse manner, the limitations metioned above tend not to substantially jeopardize what was proposed. Even though it is not possible to identify focal lesions in the heart, for example, since myocarditis due to arbovirus is diffuse, the odds are high that this form of myocarditis can be detected by puncturing random portions of the myocardium.

It is important to make it clear that use of MIA should not be encouraged at the expense of performing autopsies. Autopsy remains the gold standard but, in cases in which there is no SVO or when the family does not authorize an autopsy, MIA is an alternative.

The first MIA in Brazil was performed in São Paulo, in March 2020,[27] and later in the state of Ceará[24] and then in the state of Bahia.[28]

Confirmation of a case of human rabies in Ceará, after several years with no record of the disease in the state, was evidence of the system's increased sensitivity with the use of MIA: the family refused to authorize autopsy and the case would not have been investigated if MIA had not been performed at the hospital where the death occurred. This is, furthermore, an aspect to be discussed in this scenario of expanding the use of this technique to hospitals: i.e. whether or not MIA should be exclusively performed by pathologists. The experience of the *Universidade de São Paulo* points to the possibility of the procedure being performed by trained health professionals, even if they are not physicians. In Ceará, it was decided to only train pathologists, especially because it is they who have to provide histological diagnosis, as recommended by Brazilian legislation. In cases in which there is no SVO in the region, performance of MIA by other health professionals should be discussed and assessed. At the time of concluding this report, there is no consensus on the subject but it certainly deserves reflection, given the small number of pathologists available in the health system.

Even though the initial objective of the Ceará SVO was to use MIA in arbovirus cases, in a complementary way, indications for using MIA have been extended to other infectious diseases of public health concerrn, in order to increase the number of procedures performed by each pathologist, accelerating the learning curve of the technique and increasing sensitivity of death surveillance in Ceará.[29]

The SVO experience suggests that, when performing MIA, the corpse should be placed on a stretcher. Conventional necropsy tables have raised edges, which can make it difficult to puncture more dorsal structures, such as the kidneys. With the corpse in prone position, a suboccipital puncture is performed to collect cerebrospinal fluid from the cisterna magna. As there is no mandatory order for performing the procedure, it is recommended that each health service or professional should systemize it, so as to avoid forgetting to puncture one or more organ. The following puncture order has been established at the Ceará SVO: brain, heart, right lung, left lung, liver, spleen, right kidney and left kidney.

Regarding COVID-19 cases, it is worth mentioning that there are studies that report that corpses subjected to MIA had almost identical histological findings when compared to those subjected to autopsy.[30]

Initially, a needle was used for each organ due to the secondary objective of identifying which organ had positive laboratory results. Furthermore, the aim was to identify whether there was an organ with greater positivity than the others, with a view to prioritizing it when it was not possible to collect samples from all organs. This increases the cost of the procedure but, under normal conditions, needle consumption will be much lower.

There is no way of measuring the number of MIAs needed for a health professional to achieve a target organ success rate of 100%. The Ceará experience suggests that, with few autopsies performed, the professional has sufficient security and, if the service has ultrasound equipment and a radiologist, the MIA technique becomes more effective, facilitating completion of the process.

Historically, family members refusing to authorize autopsies has been greater in Ceará, which ends up motivating the search for less invasive alternatives; especially after the chikungunya epidemic, responsible for many deaths in elderly people whose relatives did not allow an autopsy to be performed.

Adopting MIA should not require new SVOs. Its use can expand the range of diseases observed through postmortem punctures, detection of emerging diseases and even diagnosis of chronic diseases.

The next challenge for the state of Ceará will be to train infectious disease specialists and neurologists from local reference hospitals –

Hospital São José de Ciências Infecciosas and *Hospital Geral de Fortaleza*, both located in Ceará´s capital city – to use the technique in their hospitals, in cases that are of interest to health surveillance and, especially, in cases in which family members do not allow the corpse to be sent to the Death Verification Service. Greater use of minimally invasive autopsy will certainly contribute to reducing the number of deaths with ill-defined causes in Ceará.

AUTHOR CONTRIBUTIONS

Almeida LM, Melo DN, Souza PMM, Silva FKS, Coelho TMS, Silva MM, Mota AGM, Monteiro RAA, Saldiva PHN and Cavalcanti LPG contributed to the study concept and design, analysis of the results and drafting the manuscript. Almeida LM, Melo DN, Souza PMM, Lima STS, Oliveira GGS and Cavalcanti LPG contributed to data interpretation and critically reviewing the contents of the manuscript. All the authors have approved the final version of the manuscript and are responsible for all aspects thereof, including the guarantee of its accuracy and integrity.

CONFLICTS OF INTEREST

The authors declare they have no conflicts of interest.

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RESUMO

Objetivo: *Estabelecer protocolo para realização de autópsias minimamente invasivas (AMIs) na detecção de óbitos por arboviroses e relatar dados preliminares desse protocolo no Ceará, Brasil.* ***Métodos:*** *Médicos patologistas foram treinados em AMI.* ***Resultados:*** *Estabeleceu-se protocolo para AMI, definindo-se critérios para amostras a serem coletadas, suas formas de armazenamento e diagnóstico, segundo o tipo de amostra biológica; em três meses, foram realizadas 43 AMIs, das quais 21 (48,8%) chegaram ao Serviço de Verificação de Óbito (SVO) com hipótese diagnóstica de alguma arbovirose e sete (16,3%) foram confirmados (seis de chikungunya; uma de dengue); também foram confirmados casos de covid-19 (n = 9), tuberculose (n = 5), meningite (n = 4), criptococose (n = 1), doença de Creutzfeldt-Jakob (n = 1), neoplasia de mama (n = 1) e raiva humana (n = 1).* ***Conclusão:*** *O protocolo implantado permitiu a captação de um maior número de óbitos suspeitos de arboviroses, além da confirmação de outras patologias de interesse da vigilância.*

Palavras-chave: *Infecções por Arbovirus, Autópsia, Serviços de Vigilância Epidemiológica, Técnicas de Pesquisa.*

RESUMEN

Objetivo: *Estabelecer un protocolo utilizado para la realización de autopsias mínimamente invasivas (AMI) para la detección de muertes por arbovirus y presentar datos preliminares de este protocolo en Ceará, Brasil.* ***Métodos:*** *Se llevó a cabo la capacitación de médicos patólogos en AMI.* ***Resultados:*** *Se estableció un protocolo para la realización de AMI, que define los criterios para la toma de muestras, métodos de almacenamiento y diagnóstico; en tres meses se realizaron 43 AMI; de estas, 21 (48,8%) llegaron al Servicio de Verificación de Óbito (SVO) con una hipótesis diagnóstica de alguna arbovirosis y siete (16,3%) fueron confirmadas (seis casos de chikungunya y uno de dengue); también se confirmaron casos de Covid-19 (n = 9), tuberculosis (n = 5), meningitis (n = 4), criptococosis (n = 1), enfermedad de Creutzfeldt-Jakob (n = 1), neoplasia de mama (n = 1) y rabia humana (n = 1).* ***Conclusión:*** *El protocolo implementado permitió la identificación de un mayor número de muertes sospechosas de arbovirus, además de la confirmación de otras patologías de interés.*

Palabras clave: *Infecciones por Arbovirus, Autopsia, Servicios de Vigilancia Epidemiológica, técnicas de Investigación.*

5.3. Capítulo 3 (artigo publicado)

Post-Mortem Diagnosis of Pediatric Dengue Using Minimally Invasive Autopsy during the COVID-19 Pandemic in Brazil

Tropical Medicine and Infectious Diseases

Tropical Medicine and Infectious Disease

MDPI

Case Report

Post-Mortem Diagnosis of Pediatric Dengue Using Minimally Invasive Autopsy during the COVID-19 Pandemic in Brazil

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Abstract: We report the first pediatric disease in which the use of minimally invasive autopsy (MIA) confirmed severe dengue as the cause of death. During the COVID-19 pandemic, a previously healthy 10-year-old girl living in north-eastern Brazil presented fever, headache, diffuse abdominal pain, diarrhoea, and vomiting. On the fourth day, the clinical symptoms worsened and the patient died. An MIA was performed, and cores of brain, lungs, heart, liver, kidneys, and spleen were collected with 14G biopsy needles. Microscopic examination showed diffuse oedema and congestion, pulmonary intra-alveolar haemorrhage, small foci of midzonal necrosis in the liver, and tubular cell necrosis in the kidneys. Dengue virus RNA and NS1 antigen were detected in blood and cerebrospinal fluid samples. Clinical, pathological, and laboratory findings, in combination with the absence of other lesions and microorganisms, allowed concluding that the patient had died from complications of severe dengue.

Keywords: severe dengue; autopsy; minimally invasive autopsy; arbovirus; COVID-19

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1. Introduction

Dengue is the most important arbovirus worldwide, causing epidemics with a high human health and economic impact. Severe symptoms mainly affect the pediatric population from endemic low- and middle-income countries [1,2].

In Brazil, dengue remains the most widespread disease caused by arbovirus, even after the introduction of Zika and chikungunya. In north-eastern Brazil, deaths from dengue are frequent, even in non-epidemic years, especially in socially vulnerable populations [1–3]. Clinically, most dengue infections are either asymptomatic or produce mild disease [1–3]. However, given the high number of infections, severe cases are often reported during epidemics and represent a challenge for diagnosis and clinical management. In fatal

cases, most organs and systems are affected, particularly the heart, central nervous system, gastrointestinal tract, and kidneys.

After the establishment of Death Verification Services (DVS) in Brazil, the use of conventional autopsy (CA), the gold standard technique for the diagnosis of deaths caused by dengue, has contributed to the detection of patients clinically not diagnosed [4] and has significantly reduced neglected and underreported cases. However, the existence of few DVS in the cities, together with the low acceptability of CA among the relatives of the deceased and the lack of financial resources and specialised personnel, has resulted in limited implementation of this procedure [5]. Thus, the application of new strategies for post-mortem tissue collection is necessary, particularly for pediatric deaths, as rejection of CA by the relatives is very high in this population [5–10].

Minimally invasive autopsy (MIA) has been used as an alternative to CA with promising results [8,10–14]. This technique allows obtaining core biopsies of key organs by percutaneous puncture, with or without guidance with an imaging technique. MIA has been widely used in the context of the COVID-19 pandemic as a fast and non-disfiguring method with minimal biological risk for the personnel performing the procedure [8,9,15–17]. However, current knowledge of the performance of this technique for arboviral diseases in the paediatric population is very limited.

We report the first case of fatal dengue infection, which occurred in a previously healthy 10-year-old girl living in north-eastern Brazil during the COVID-19 pandemic. MIA sampling allowed correct diagnosis and showed complete agreement with the CA. We show that this acceptable, simplified, and non-disfiguring post-mortem technique can reliably diagnose death from severe dengue.

2. Case Report

A 10-year-old girl presented with fever, headache, diffuse abdominal pain, diarrhoea, and vomiting at the end of June 2021. She was previously healthy and had no comorbidities. A previously healthy 10-year-old girl with no comorbidities presented with fever, headache, diffuse abdominal pain, diarrhoea, and vomiting at the end of June 2021. The patient was initially treated with dipyrone. After 24 h, the patient presented dark stools. Two days later her clinical condition worsened and she was admitted to an emergency care unit (ECU), in which a blood count revealed thrombocytopenia (57,000/mm^3). Intravenous hydration, antipyretics, and antiemetics were administered. After 3 days, the abdominal pain worsened, and the patient developed cutaneous pallor, arterial hypotension, and drowsiness, and was transferred to a paediatric hospital, where she arrived pale, with cold skin, thin pulse, gasping, dehydrated, and with tense abdomen. Myocarditis was considered by the physician. A femoral central venous access allowed expansion with albumin. The blood count revealed mild anaemia (haemoglobin 11.9 g/dL, haematocrit 35.9%), lymphopenia (92/mm^3), and thrombocytopenia (57,000/mm^3 on admission, which dropped to 20,000/mm^3 within a few hours). Liver enzymes were above reference levels during hospitalisation (aspartate aminotransferase 741.1 U/L; alanine aminotransferase 248.9 U/L). She also had altered renal function, hyperkalaemia (10 mmol/L), and severe metabolic acidosis (pH 6.7). Activated partial thromboplastin time and prothrombin time were prolonged (Table 1). The next day, the patient suffered cardiorespiratory arrest, unresponsive to resuscitation measures. There was profuse bleeding through the oropharynx, trachea, and stomach. The clinical diagnoses were severe acute hepatitis of unexplained cause, acute renal dysfunction, and shock.

The mother of the patient reported the presence of several neighbors with similar symptoms and the recent admission of an aunt who lived with her, who had been diagnosed with severe dengue. Neither respiratory symptoms nor recent contact with suspected or confirmed cases of COVID-19 were described. Remarkably, co-circulation of SARS-CoV-2 and dengue has recently been reported in the Americas [18].

Table 1. Results of laboratory tests.

Exam	27 June 2021 (3 Days of Symptom)	28 June 2021 (4 Days of Symptom)	Reference Values
Red Cells	4.29 million/mm^3	4.11 million/mm^3	4.1 to 5.3 million/mm^3
Haemoglobin	12.3 g/dL	11.9 g/dL	12 to 14.5 g/dL
Haematocrit	35.9%	35%	36 to 43%
Leukocytes	2300/mm^3	5600/mm^3	3400 to 10,800/mm^3
Neutrophils	1955/mm^3	4424/mm^3	1500 to 8500/mm^3
Rod Neutrophils	69/mm^3	224/mm^3	0 to 860/mm^3
Segmented Neutrophils	1886/mm^3	4200/mm^3	1500 to 8500/mm^3
Eosinophils	0/mm^3	56/mm^3	0 to 500/mm^3
Lymphocytes	92/mm^3	672/mm^3	1500 to 6500/mm^3
Monocytes	253/mm^3	336/mm^3	0 to 800/mm^3
Basophils	0/mm^3	0/mm^3	0 to 200/mm^3
Platelets	57,000/mm^3	20,000/mm^3	150 to 450 mil/mm^3
Atypical Lymphocytes	-	112/mm^3	0%
MPV	8.3 fL	7.5 fL	9.2 to 12.6 fL
Ultrasensitive C-reactive protein	2.96 mg/dL	2.37 mg/dL	<0.10 mg/dL
Magnesium	-	2.02 mg/dL	2.02 to 2.75 mg/dL
Potassium	4.0 mmol/L	6.8 mmol/L	3.5 to 5.1 mmol/L
Sodium	136 mmol/L	139 mmol/L	136 to 145 mmol/L
AST	83.3 U/L	741.1 U/L	17 to 33 U/L
ALT	27.3 U/L	248.9 U/L	9 to 23 U/L
Urinary Urobilinogen	3.0 mg/dL	-	< 1.0 mg/dL
Creatinine	-	0.82 mg/dL	0.32 to 0.61 mg/dL
Urea	-	28.9 mg/dL	19.2 to 46.2 mg/dL
TAP—prothrombin time	-	16.8 s	9.4 to 12.5 s
APTT- activated partial thromboplastin	-	49.8 s	25.1 to 36.5 s
	Laboratory tests performed after death		
Blood culture			No microbial growth
RT-PCR for SARS-CoV-2			Not Detectable
qRT-PCR for dengue			Positive
qRT-PCR for Zika			Negative
qRT-PCR for chikungunya			Negative
NS1 antigen			Positive

Subtitle: MPV—mean platelet volume, AST—aspartate aminotransferase, ALT—alanine aminotransferase, TAP—prothrombin time, APTT—activated partial thromboplastin, RT-PCR—reverse transcriptase-polymerase chain reaction, qRT-PCR—quantitative reverse transcriptase-polymerase chain reaction.

The body was sent to the DVS Dr Rocha Furtado (DVS-RF), where an MIA followed by CA were performed, after consent provided by the mother. The post-mortem procedures were performed as part of a study approved by the Research Ethics Committee through protocol CAAE 27162619.1.0000.5049, number 3,851,684.

Nasopharyngeal swabs were routinely tested by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) for SARS-CoV-2 in all DVS-RF autopsies performed during the COVID-19 pandemic. About 20 mL of blood and 2 mL of cerebrospinal fluid (CSF) were collected as part of the MIA procedure before the CA. The two post-mortem procedures were analysed by two different pathologists.

For the MIA, 20 cm, 14 Gauge percutaneous biopsy needles were used. Four brain cores (1.2 to 1.3 cm) were obtained by introducing the biopsy needle through the right and left nasal cavity, piercing the cribriform plate of the sphenoid bone. The right and the left lungs were punctured between the third and fourth intercostal spaces, and four cores from each lung (0.5 to 0.6 cm from the right lung; 1.2 to 1.4 cm from left lung) were collected. Four cores (0.8 to 0.9 cm) were obtained from the heart, after puncture in the fifth intercostal space. The liver was punctured in the right 11th intercostal space, in the anterior axillary line, and four tissue cores (0.7 to 1.0 cm) were obtained. Punctures directed to the kidneys were performed in the right and left subcostal spaces and four tissue fragments from each side were obtained (1.5 to 1.7 cm right, 1.0 to 1.2 cm left). Finally, four cores were collected from the splenic area (0.8 to 1.0 cm).

CA was performed following the DVS-RF protocol (4), after opening all cavities. The brain was swollen (weight 1310 g). Bilateral pleural effusion and ascites were observed. The lungs were oedematous and showed areas of haemorrhage (weight: 365 g left and 375 g right). The liver and the spleen showed congestion and weighed 1200 g and 415 g, respectively. One-hundred-and-fifty milliliters of fresh blood were identified in the stomach. The kidneys were pale and oedematous (weight: 110 g right and 100 g left). The adrenals showed no abnormalities.

Microscopic examination showed oedema and congestion in all organs, foci of intra-alveolar haemorrhage in the lungs, and foci of midzonal necrosis in the liver. Hypoplasia of the white pulp of the spleen was associated with abundant macrophages with large clear nuclei. There was extensive coagulative necrosis of the cortical tubules of the right kidney.

Samples of the left kidney and spleen of the MIA showed only skeletal muscle, connective tissue, vessels, and nerves under microscopy, with no cores of parenchyma.

The nasopharyngeal swab, blood, and CSF were sent to the Central Laboratory of Public Health of Ceará (LACEN-CE) for laboratory tests: qRT-PCR for respiratory viruses, arboviruses (dengue, Zika, and chikungunya), and detection of dengue NS1 antigen in blood and CSF [19]. A blood culture for bacterial research was also performed.

The following findings were of note: midzonal hepatocyte necrosis with rare acidophilic bodies seen only in the MIA samples, which were better preserved; enlargement of the alveolar septa by inflammatory cells (viral interstitial pneumonitis), edema and foci of intraalveolar hemorrhage seen in both MIA and CA; and acute tubular necrosis in the kidneys (Figure 1). Previous studies reporting histological findings in fatal cases of dengue have reported similar changes, including diffuse congestion and hemorrhage, alveolar edema, and liver cell necrosis [20].

The nasopharyngeal swab sample tested negative for SARS-CoV-2 RNA and there was no microbial growth in the blood culture. The qRT-PCR test for arboviruses identified the presence of DENV-2 RNA in the blood sample, and the NS1 antigen (kit J. Mitra & Co. Pvt. Ltd.) tests were positive for dengue in the blood and CSF samples [21] All tests performed for Zika and chikungunya viruses were negative (Table 1).

Clinical features, such as upper digestive and pulmonary hemorrhage, acute tubular necrosis, and shock causing death, in conjunction with the pathological and laboratory findings, were in keeping with the diagnosis of death due to complications of severe dengue. Remarkably, the samples collected by the MIA in this pediatric patient were sufficient to confirm the diagnosis of severe dengue and were completely in agreement with the samples collected by the CA (Figure 1).

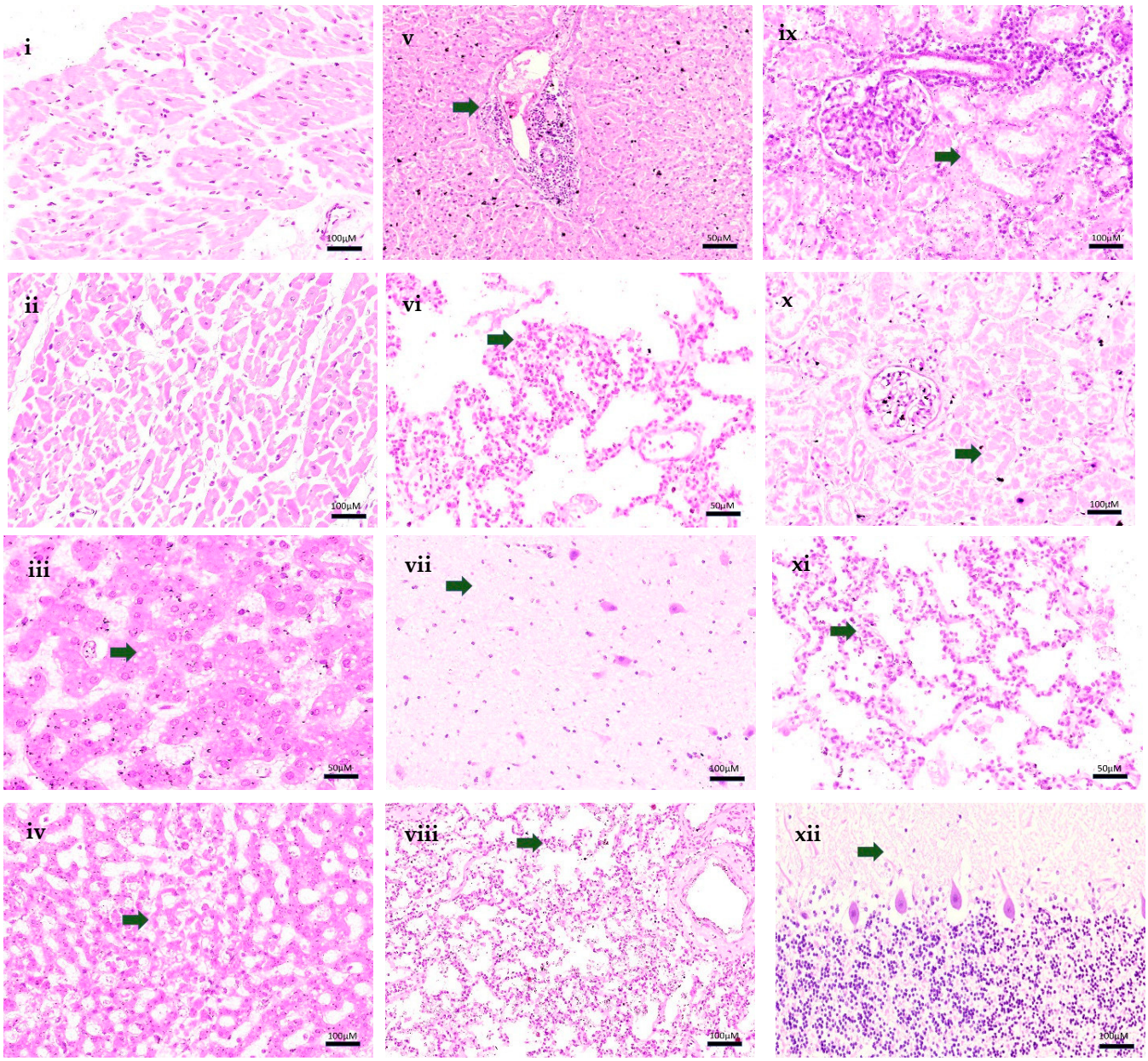

Figure 1. Images of MIA and CA samples of patient. (**i**)—Heart. Cardiac muscle fibres. (HE, 100X—MIA). (**ii**)—Heart. Cardiac muscle fibres. (HE, 100X—CA). (**iii**)—Liver. Mild microvesicular steatosis. (HE, 50X—MIA). (**iv**)—Liver. Midzonal necrosis of hepatocytes. (HE, 100X—MIA). (**v**)—Liver. Mononuclear portal infiltrate. Edema and congestion. (HE, 50X)—CA). (**vi**)—Lung. Interstitial pneumonitis. (HE, 50X—MIA). (**vii**)—Lung. Interstitial pneumonitis. (HE, 100X—CA). (**viii**)—Lung. Interstitial pneumonitis. Alveolar edema. (HE, 100X—CA). (**ix**)—Kidney R. Necrosis of renal tubules. (HE, 100X—MIA). (**x**)—Kidney Necrosis of renal tubules. (HE, 100X—CA). (**xi**)—Brain. Brain edema. (HE, 50X—MIA). (**xii**)—Cerebellum. Edema. (HE, 100X—CA). Legend: CA = conventional autopsy; MIA = minimally invasive autopsy.

3. Conclusions

Disease surveillance and patient healthcare requires adequate ascertainment of the cause of death, especially in the current context of circulation of multiple arboviruses and other pathogens with the potential of causing epidemics. In a scenario of reduced acceptability of CA, MIA is a promising tool, which has proven to be successful even during the COVID-19 pandemic, for diagnosing arboviral-related deaths.

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6. CONSIDERAÇÕES FINAIS

O fluxo para realização de autopsias minimamente invasivas para a vigilância de óbitos por arboviroses foi implantado no Serviço de Verificação de Óbitos Dr. Rocha Furtado, no Ceará.

A aplicação do fluxo de AMI se mostrou capaz de ampliar o número de óbitos suspeitos de arboviroses investigados. Possibilitou, ainda, o diagnóstico de outras doenças de interesse da vigilância epidemiológica, sobretudo as de notificação compulsória.

O sucesso das punções (obtenção de amostra de tecido e tecido correspondendo ao orgão de interesse) variou entre 18 e 30% para baço e rim esquerdo. Foi de 100% para sangue, LCR, pulmão, fígado e cérebro. A quantidade de tecido obtido no procedimento variou de menos de 10mm² para pulmão, baço e rim, até mais de 35mm² para fígado e cérebro.

Através do treinamento em serviço realizado no SVO-RF, um patologista consegue realizar a coleta de fluidos e tecidos, por meio da AMI, utilizando um fluxo simplificado.

A autorização dos familiares pareceu ser mais facilmente obtida para a AMI do que para autopsia convencional.

A utilização da AMI, isoladamente, pode reduzir o tempo de permanência do corpo no SVO-RF.

A autopsia convencional permanece sendo o padrão-ouro, mas nos casos em que não é viável, a AMI cumpre um papel importante de prover amostras biológicas de qualidade para investigação etiológica e esclarecimento da causa da morte.

O uso do ultrassom pode contribuir, substancialmente, para o sucesso na obtenção de amostras dos órgãos-alvo. Contudo, nem todos os profissionais se mostram seguros na utilização do equipamento.

Um médico bem treinado é capaz de colher material biológico para investigação da causa de morte em ambientes tais como num hospital, nos casos em que a família não permite o deslocamento do corpo para o SVO, bem como quando o serviço não esteja acessível.

A rejeição de alguns profissionais do SVO-RF para utilizar o fluxo de coleta e realizar AMI, configurou-se como uma limitação ao estudo. As causas para essa rejeição precisam ser investigadas.

Mostra-se oportuno ampliar a discussão sobre a terminologia mais adequada, considerando o cenário de rejeição ao termo: "*autopsia*". Há grupos que preferem chamar a técnica de Minimally Invasive Tissue Sampling - **MITS** (amostragem de tecido minimamente invasiva), o que pode ser mais adequado para sensibilização de profissionais e familiares.

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ANEXO A – ORGANIZAÇÃO DA SALA DE AUTÓPSIAS NO SVO

Preparação da sala de autópsia no SVO-RF, para realização da AMI durante a pesquisa.

ANEXO B - APROVAÇÃO DO COMITÊ DE ÉTICA

CENTRO UNIVERSITÁRIO
CHRISTUS - UNICHRISTUS

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: AVALIAÇÃO DO IMPACTO DA UTILIZAÇÃO DA TÉCNICA DE AUTOPSIA MINIMAMENTE INVASIVA (MINIMALLY INVASIVE AUTOPSY - MIA) COMO FORMA DE AMPLIAR A SENSIBILIDADE DA VIGILÂNCIA DE ÓBITOS POR ARBOVIROSES NO CEARÁ, NORDESTE DO BRASIL

Pesquisador: LUCIANO PAMPLONA DE GOES CAVALCANTI

Área Temática:

Versão: 1

CAAE: 27162619.1.0000.5049

Instituição Proponente: Instituto para o Desenvolvimento da Educação Ltda-IPADE/Faculdade

Patrocinador Principal: MINISTERIO DA SAUDE

DADOS DO PARECER

Número do Parecer: 3.851.684

Apresentação do Projeto:

Introdução: a tripla circulação simultânea das arboviroses dengue, Zika e chikungunya se tornou um grave problema de saúde pública no Brasil, especialmente nas regiões nordeste e sudeste do País, onde são registrados muitos óbitos por essas doenças. A investigação desses óbitos suspeitos é obrigatória, mas apesar do avanço na vigilância de óbitos no Brasil e dos investimentos feitos com a ampliação da rede de Serviços de Verificação de Óbitos (SVO), a condição da vigilância para determinar a causa dos óbitos por arboviroses permanece como um importante desafio para as políticas públicas. No Estado do Ceará, há óbitos registrados por dengue desde 1994, apresentando uma das maiores letalidades do Brasil e com a epidemia recente de chikungunya, ocorrida em 2016/17, foram registrados mais óbitos que em 30 anos de dengue somados, evidenciando o impacto potencial da circulação simultânea dessas arboviroses. No Ceará, mesmo com a grande contribuição do SVO para elucidação da causa de vários óbitos através das autópsias, uma das limitações tem sido a recusa de familiares para realização do procedimento, principalmente nos casos em que envolve a população mais idosa. O mesmo cenário epidemiológico, mas com circulação mais recente desses arbovírus, é detectado na cidade de Campinas, SP. Em consonância com essa situação, há um interesse crescente no mundo em desenvolver técnicas menos invasivas, potencialmente mais aceitáveis, e que consigam fornecer

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informações conclusivas sobre a causa dos óbitos. A autópsia completa continua sendo o padrão ouro na determinação da causa de morte. No entanto, realizar essas autópsias em locais que não tenham SVO articulado com a vigilância epidemiológica, ou ainda, nos casos em que a família não permite a realização dessa autopsia tem sido um desafio para o sistema de saúde. Nesse cenário surge a possibilidade de uso da autópsia minimamente invasiva (Minimally Invasive Autopsy - MIA), uma abordagem inovadora para obter amostras post-mortem de órgãos-chave, que é cada vez mais reconhecida como uma metodologia robusta para investigação sobre a causa da morte. Objetivo: avaliar o impacto da utilização da Autopsia Minimamente Invasiva (Minimally Invasive Autopsy - MIA) para vigilância de óbitos suspeitos por arbovírus (dengue, Zika e chikungunya) no estado do Ceará, provendo uma técnica capaz de aumentar a capacidade do SUS de investigar e encerrar esses óbitos. Métodos: trata-se de um estudo prospectivo analítico
3
envolvendo óbitos suspeitos de arbovírus (dengue, zika e chikungunya), autopsiados nos Serviço de Verificação de Óbitos de Fortaleza, entre os anos de 2020 e 2022, que comtemplem uma dessas situações: a) óbitos que forem encaminhados para o SVO, como suspeitos de dengue e/ou zika e/ou chikungunya OU b) óbitos que forem encaminhados com outra hipótese diagnóstica e os patologistas do SVO suspeitem de alguma arbovirose, como causa provável da morte. Essa suspeita, por parte dos patologistas, deverá ocorrer em dois momentos distintos: a) após a entrada do corpo no SVO e antes da realização da autopsia, por meio de uma entrevista que é realizada por um médico patologista ou clínico, com os familiares OU b) durante a realização da autopsia, nos casos em que: I - houver registro de febre recente (máximo sete dias), sem infecção bacteriana aparente ou, II - nos casos em que for detectada a presença de exantema, derrames cavitários ou hemorragia ou III - corpos que forem encaminhados com hipótese diagnóstica de doenças que fazem diagnóstico diferencial com essas arboviroses. Os corpos serão identificados, será preenchido um perfil socioeconômico e após aprovação da família e assinatura do TCLE serão realizados os procedimentos (autopsia completa e/ou MIA). As amostras de vísceras e líquidos serão devidamente armazenadas e enviadas para o laboratório para análise. Todas as amostras serão testadas para dengue, zika e chikungunya usando as técnicas ELISA IgM e IgG, RT-PCR, isolamento viral, imunohistoquímica e histopatológico. Nos casos em que a família não autorizar a realização da autopsia convencional completa será solicitado autorização para realização da MIA. A concordância na categoria de doença obtida pelos dois métodos será avaliada pelo método de Kappa. Este estudo seguirá os princípios éticos de pesquisa envolvendo seres humanos, conforme Resolução 466/12, do Conselho Nacional de Saúde, respeitando os princípios fundamentais de

autonomia, beneficência, não maleficência, justiça e equidade.A tripla circulação, simultânea, das arboviroses dengue, zika e chikungunya se tornou um grave problema de saúde pública no Brasil, especialmente na região nordeste do País, onde são registrados muitos óbitos (CARVALHO & CAVALCANTI, 2016; FREITAS et al, 2017; BRITO, 2016).

No Brasil, a investigação de óbitos suspeitos de arboviroses é obrigatória (Brasil, 2016). Recomenda-se investigar oportunamente todo óbito de caso suspeito, visando identificar as causas e propor intervenções que evitem novos óbitos. Esta investigação deve ser iniciada imediatamente após a ocorrência do óbito (BRASIL, 2016). Entretanto, apesar do avanço na vigilância de óbitos no Brasil e dos investimentos feitos com a ampliação dos Serviços de Verificação de Óbitos (SVO), a condição da vigilância para determinar a causa dos óbitos permanece como um importante desafio para as políticas públicas.

A Portaria ministerial 1405/2006 criou a Rede Nacional de Serviços de Verificação de Óbitos (SVO), com a finalidade de esclarecer a causa mortis em casos de morte natural, sem assistência médica, e os de causas não determinadas ou mal definidas em vida. Também considera a importância de elucidar a causa mortis, especialmente aqueles casos de interesse da vigilância epidemiológica.

Nessa perspectiva a autópsia e os estudos pós-morten aumentaram a compreensão de como os microorganismos podem causar doenças em hospedeiros humanos, especialmente em doenças infecciosas emergentes e reemergentes, provando ser instrumento patológico relevante. No Estado do Ceará, há óbitos registrados por dengue desde 1994, apresentando uma das maiores letalidades do Brasil, com registro de uma grande série histórica de óbitos (CAVALCANTI et al, 2018; MONTEIRO et al, 2019). Entretanto, já durante a primeira epidemia de Chikungunya, ocorrida em 2016/17, foram registrados mais óbitos que em 30 anos de dengue somados, evidenciando o impacto potencial da circulação simultânea dessas arboviroses (CAVALCANTI et al, 2019; SIMIÃO et al, 2019).

Objetivo da Pesquisa:

IVOS

4.1 Geral

Avaliar o impacto da utilização da Autopsia Minimamente Invasiva (Minimally Invasive Autopsy - MIA) para vigilância de óbitos suspeitos por arbovírus (dengue, zika e chikungunya) no estado do Ceará, provendo uma técnica capaz de aumentar a capacidade do SUS de investigar e encerrar esses óbitos.

4.2 Específicos Ampliar o número de óbitos suspeitos de arbovírus investigados, melhorando a capacidade de diagnóstico da vigilância epidemiológica. Medir a sensibilidade da técnica de

autopsia minimamente invasiva (MIA) quando comparada a autopsia convencional completa para o diagnóstico de arboviroses. Avaliar a aceitabilidade e percepção da técnica de autopsia minimamente invasiva (MIA) por profissionais de saúde no ambiente hospitalar e SVO, além dos familiares desses óbitos. Estimar o custo da realização de uma necropsia utilizando a técnica de autopsia minimamente invasiva (MIA), para o serviço de saúde. Comparar se o tempo entre o óbito e a realização dos procedimentos (MIA x autopsia completa convencional) interfere na qualidade das amostras coletadas e na sensibilidade do diagnóstico final. Avaliar se alterações moleculares nas amostras dos tecidos são mais intensas em tecidos e indivíduos em que ainda há replicação viral ativa. Buscar alterações moleculares que apontem assinatura gênica que marca gravidade e sugerir receptores, ou vias, que possam ser utilizadas no desenvolvimento de novas terapias.

Avaliação dos Riscos e Benefícios:

Como se trata de coleta de material biológico de paciente após confirmação do óbito o desconforto é emocional para o familiar, no sentido de autorizar a autopsia. Esclarecemos ainda que, nos casos de doença infecciosa de interesse da vigilância epidemiológica a autopsia é indicada. Nesse sentido, a assistente social do SVO orienta as famílias sobre os procedimentos a serem realizados antes, durante e após a realização da autopsia.

O principal benefício será de contribuir para o conhecimento dos órgãos públicos sobre características das epidemias de chikungunya, dengue e zika no município e um benefício individual das pessoas que participarem do estudo que terão acesso ao seu diagnóstico em relação a essas arboviroses.

Comentários e Considerações sobre a Pesquisa:

Pertinente

Considerações sobre os Termos de apresentação obrigatória:

presentes

Recomendações:

sem recomendações

Conclusões ou Pendências e Lista de Inadequações:

sem pendencia

ja tem patrocinio

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Continuação do Parecer: 3.851.684

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_BÁSICAS_DO_PROJETO_1480539.pdf	18/12/2019 17:30:36		Aceito
Declaração de Instituição e Infraestrutura	_CARTA_APOIO_UFC.pdf	18/12/2019 17:29:13	LUCIANO PAMPLONA DE GOES CAVALCANTI	Aceito
Orçamento	orcamento_CEP_MIA.pdf	29/11/2019 10:43:48	LUCIANO PAMPLONA DE GOES CAVALCANTI	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_MIA.pdf	29/11/2019 10:43:33	LUCIANO PAMPLONA DE GOES CAVALCANTI	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_MIA_CEP_3.pdf	29/11/2019 10:43:06	LUCIANO PAMPLONA DE GOES CAVALCANTI	Aceito
Folha de Rosto	folhaderosto.pdf	27/11/2019 18:26:43	LUCIANO PAMPLONA DE GOES CAVALCANTI	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

FORTALEZA, 20 de Fevereiro de 2020

Assinado por:
OLGA VALE OLIVEIRA MACHADO
(Coordenador(a))

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ANEXO C – OUTROS ARTIGOS PUBLICADOS DURANTE O DOUTORADO

Clinical Infectious Diseases

MAJOR ARTICLE

Fatal Outcome of Chikungunya Virus Infection in Brazil

Shirlene Telmos Silva de Lima,[1,2,a] William Marciel de Souza,[3,a] John Washington Cavalcante,[1,a] Darlan da Silva Candido,[4,a] Marcilio Jorge Fumagalli,[3,a] Jean-Paul Carrera,[4,5] Leda Maria Simões Mello,[2] Fernanda Montenegro de Carvalho Araújo,[2,6] Izabel Letícia Cavalcante Ramalho,[2] Francisca Kalline de Almeida Barreto,[1] Deborah Nunes de Melo Braga,[7] Adriana Rocha Simião,[1] Mayara Jane Miranda da Silva,[8] Rhaquel de Morais Alves Barbosa Oliveira,[1] Clayton Pereira Silva Lima,[8] Camila de Sousa Lins,[6] Rafael Ribeiro Barata,[8] Marcelo Nunes Pereira Melo,[6] Michel Platini Caldas de Souza,[8] Luciano Monteiro Franco,[6] Fábio Rocha Fernandes Távora,[6] Daniele Rocha Queiroz Lemos,[6] Carlos Henrique Morais de Alencar,[1] Ronaldo de Jesus,[9] Vagner de Souza Fonseca,[9,10] Leonardo Hermes Dutra,[10] André Luiz de Abreu,[10] Emerson Luiz Lima Araújo,[10] André Ricardo Ribas Freitas,[11] João Lídio da Silva Gonçalves Vianez Júnior,[8] Oliver G. Pybus,[4] Luiz Tadeu Moraes Figueiredo,[3] Nuno Rodrigues Faria,[4,12,b] Márcio Roberto Teixeira Nunes,[8,b] Luciano Pamplona de Góes Cavalcanti,[1,b] and Fabio Miyajima[1,13,b]

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Background. Chikungunya virus (CHIKV) emerged in the Americas in 2013 and has caused approximately 2.1 million cases and >600 deaths. A retrospective investigation was undertaken to describe clinical, epidemiological, and viral genomic features associated with deaths caused by CHIKV in Ceará state, northeast Brazil.

Methods. Sera, cerebrospinal fluid (CSF), and tissue samples from 100 fatal cases with suspected arbovirus infection were tested for CHIKV, dengue virus (DENV), and Zika virus (ZIKV). Clinical, epidemiological, and death reports were obtained for patients with confirmed CHIKV infection. Logistic regression analysis was undertaken to identify independent factors associated with risk of death during CHIKV infection. Phylogenetic analysis was conducted using whole genomes from a subset of cases.

Results. Sixty-eight fatal cases had CHIKV infection confirmed by reverse-transcription quantitative polymerase chain reaction (52.9%), viral antigen (41.1%), and/or specific immunoglobulin M (63.2%). Co-detection of CHIKV with DENV was found in 22% of fatal cases, ZIKV in 2.9%, and DENV and ZIKV in 1.5%. A total of 39 CHIKV deaths presented with neurological signs and symptoms, and CHIKV-RNA was found in the CSF of 92.3% of these patients. Fatal outcomes were associated with irreversible multiple organ dysfunction syndrome. Patients with diabetes appear to die at a higher frequency during the subacute phase. Genetic analysis showed circulation of 2 CHIKV East-Central-South African (ECSA) lineages in Ceará and revealed no unique virus genomic mutation associated with fatal outcome.

Conclusions. The investigation of the largest cross-sectional cohort of CHIKV deaths to date reveals that CHIKV-ECSA strains can cause death in individuals from both risk and nonrisk groups, including young adults.

Keywords. chikungunya virus; *Alphavirus*; arthritogenic; arbovirus; fatal cases.

Chikungunya virus (CHIKV) is an enveloped, single-stranded positive-sense RNA virus that belongs to the *Alphavirus* genus, Togaviridae family [1]. It is mainly transmitted to humans by the *Aedes aegypti* and *Aedes albopictus* mosquitoes. Most cases are characterized by an acute infection with fever, myalgia, exanthema, and arthralgia lasting up to 3 weeks postinfection [2, 3]. For some CHIKV cases, arthralgia can persist for >3 months, indicating a transition to a chronic stage [2, 3].

In July 2014, the first autochthonous chikungunya cases were reported in Brazil. Genetic analysis revealed the co-circulation of 2 distinct genotypes introduced almost simultaneously in the country. CHIKV Asian genotype was detected in Amapá state, North Brazil and the CHIKV East-Central-South African (ECSA) genotype was first reported in Bahia state, Northeast [4]. Until December 2019, >800 000 CHIKV cases were notified in all regions of Brazil [5].

Between 2013 and 2019, the Pan American Health Organization reported 631 deaths associated with CHIKV infection in South America, likely an underestimation of deaths caused by CHIKV [6]. The highest number of CHIKV-related deaths in the Americas, 214 deaths, was reported in the Ceará state, Northeast Brazil, where an Arbovirus Death Investigation

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[a]S. T. S. L., W. M. S., J. W. C., D. S. C., and M. J. F. contributed equally to this work.

[b]N. R. F., M. R. T. N., L. P. G. C., and F. M. contributed equally to this work.

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Committee was created due to the high mortality rate and increase of suspected arbovirus-associated deaths [6–8]. This high number of reported fatalities is comparable to what was observed in La Reunion Island [9, 10], where the outbreak was caused by the CHIKV Indian Ocean lineage [11]. In addition, neurologic infections and fatal cases were previously reported during the CHIKV epidemic in 1963–1965 in India [12–14].

Despite their importance for public health management, epidemiological and clinical investigations of fatal cases caused by CHIKV typically focus on case reports [15–21]. Moreover, no information exists on the genetic diversity of CHIKV circulating in Ceará state. This study summarizes the demographic, clinical, laboratorial, and postmortem findings of the largest cross-sectional cohort of CHIKV deaths to date.

MATERIALS AND METHODS

Study Population and Ethics Statement

We selected all fatal cases with clinical suspected arboviral infection and sera, cerebrospinal fluid (CSF), and tissue samples available recorded by the Death Verification Service of State Health Secretariat of Ceará, between December 2016 and October 2017. In each case, autopsies were undertaken within 12 hours postmortem. Sera, CSF, and tissue samples were collected and stored at −80°C for subsequent investigation. Tissues were also conserved in formalin-fixed blocks, and were sent to Evandro Chagas Institute and the Brazilian Ministry of Health for arboviral histopathological and immunohistochemistry (IHC) analyses. The study was conducted after approval by the ethics committee of the Federal University of Ceará (CAAE number: 85921418.3.0000.5054), Brazil.

Case Definitions

A suspected fatal case of CHIKV infection was defined as a patient that presented with fever, rash, arthralgia, headache, and/or myalgia during the hospitalization or perimortem period. A confirmed CHIKV death was defined as a suspected fatal case associated with CHIKV infection, plus 1 positive laboratory result for CHIKV either by reverse-transcription quantitative polymerase chain reaction (RT-qPCR), immunoglobulin M (IgM) detection, and/or IHC [5]. In addition, we defined an acute fatal outcome as a case that lasted up to 20 days, representing the acute disease; and a subacute fatal outcome, as a case that lasted longer than 20 days up to 90 days. Patients with neurologic manifestations (eg, confusion and syncope) and RT-qPCR or IgM detection against CHIKV in CSF were considered to have a central nervous system infection caused by CHIKV [5].

Laboratory Testing

RNA from CSF and brain tissue was extracted using the QIAamp Viral RNA Mini Kit (Qiagen) and the ReliaPrep RNA Miniprep Systems (Promega), respectively. Extracted RNA was tested by specific RT-qPCR for CHIKV [22], DENV [23], and ZIKV [24]. We also tested the postmortem sera and CSF samples using IgM-capture enzyme-linked immunosorbent assays (ELISAs) for antibody response against CHIKV (Euroimmun), DENV (Serion), and ZIKV (NovaTec Immundiagnostica GmbH). In addition, an ELISA for DENV-NS1 antigens (Bio-Rad Laboratories) was used. We also performed IHC analysis of liver and brain tissue samples of all patients [25].

Based on the laboratory diagnosis from the investigation of fatal cases of CHIKV, complete clinical records were obtained from the laboratory management system (Gerenciador de Ambiente Laboratorial [GAL]). Autopsy records for confirmed CHIKV fatal cases were obtained from the Death Verification Service (www.saude.ce.gov.br). Demographic, epidemiological, and clinical data (including comorbidities/immunosuppression history) were extracted from GAL, autopsy records, and home visit reports with the family of patients who died.

Epidemiological Data

Epidemiological analysis of autochthonous CHIKV cases in Ceará state was undertaken using weekly reports of suspected cases from January to December 2017. Epidemiological reports of cases were obtained from the Brazilian Ministry of Health. Incidences were calculated based on the estimated population of Ceará state in 2017, as reported by the Brazilian Institute of Geography and Statistics (www.ibge.gov.br).

Statistical Analysis

Descriptive analyses of results are reported as frequencies, arithmetic means, and ranges (when appropriate). The Mann-Whitney *U* test performed in RStudio 1.2.1335 (www.rstudio.com), was used to compare groups with comorbidities. For the regression analysis, the outcome variable was classified as patients with fatal outcome during the acute or subacute phase of the disease. The most frequent comorbidities in our cohort were used for statistical analysis. To determine comorbidities that may affect the frequency of death during the acute or subacute phase of the disease, we conducted a univariate logistic regression analysis, followed by a multivariable logistic regression controlling for sex and age. *P* values with $\alpha < .05$ were considered to be significant. Statistical analyses were performed with the Stata version 14 software (StataCorp).

Viral Genomic Sequencing and Phylogenetic Analysis

Genome sequencing was performed using a targeted multiplex PCR scheme that can amplify both CHIKV genotypes circulating in Brazil [26]. This was followed by Nanopore genome sequencing using the MinION sequencing platform (Oxford Nanopore Technologies) [26]. Preliminary runs showed that all sequences belonged to the same genotype. To improve consensus sequencing coverage, we selected 36 publicly available full genome strains and redesigned CHIKV specific sequencing primers for the genotype identified on preliminary runs by correcting mismatches. Consensus sequences were generated with

a validated bioinformatics pipeline, considering a minimum coverage depth of 20× [26].

Newly generated CHIKV consensus sequences were aligned to 87 publicly available whole-genome sequences from Brazil using MAFFT version 7.450 [27]. A maximum likelihood (ML) phylogenetic tree was estimated with RAxML version 8 [28] using the GTR+I+γ substitution model [28]. Dated phylogenetic trees were estimated using BEAST version 1.10 under a GTR+I+γ model [29]. We used a strict molecular clock model and a Skygrid tree prior [30] that were previously determined as best-fit models [29]. Evolutionary analyses were run independently in duplicate for 50 million steps, and sampling parameters and trees every 10 000 steps. Maximum clade credibility summary trees were generated using TreeAnnotator version 1.10 [29].

Data Availability

Protocols and the new sequencing primers are publicly available at caddecentre.org/protocols. Epidemiological data, phylogenetic trees, XMLs, and Ceará CHIKV genome sequences are available on the DRYAD repository (available on https://datadryad.org/stash/share/y0poMC_pufbi2DEAelhcOdJDOjFYlud_D5s56V-fMC8). New sequences have been deposited in GenBank with accession numbers MT877206-MT877211.

RESULTS

In 2017, Ceará state in Brazil experienced a major CHIKV outbreak accounting for 65.7% (n = 105 229/160 166) of suspected cases in all 27 Brazilian federal states in that year. CHIKV incidence in Ceará state was 1166 suspected cases per 100 000 inhabitants, the highest in the country [31]. Within Ceará, the municipality of Fortaleza (state capital city) accounted for the majority of CHIKV suspected cases (n = 61 825/105 229 [58.8%]) (Figure 1A). Moreover, Ceará notified a total of 194 CHIKV-related deaths in 2017 [32]. This corresponds to a case-fatality rate of 1.8 deaths per 1000 cases in 2017. Importantly, Fortaleza city reported the highest number of CHIKV-related deaths (n = 144/194 [74.2%]) in 2017 in Ceará [8]. As expected, CHIKV deaths followed a similar temporal distribution to that of the suspected cases in Ceará, with most cases reported in Fortaleza (Figure 1A and 1B).

We used RT-qPCR, serology, and IHC to ascertain the cause of death of 100 suspected arbovirus fatal cases. A total of 68% (68/100) of the cases were positive for CHIKV by at least 1 diagnostic method (Figure 1C). Of these, 70.6% (48/68) were positive by 2 or more methods. We found that 73.5% (50/68) of deaths were positive for CHIKV only, while 22% (15/68) had viral co-detection with DENV, 2.9% (2/68) co-detection with ZIKV, and 1.5% (1/68) with both DENV and ZIKV. Moreover, CHIKV-RNA was detected in the CSF of 52.9% (36/68) and in the brain of 11.1% (4/36) of the cases. Notably, no DENV or ZIKV RNA was detected in the CSF of CHIKV deaths (Figure 1C).

Figure 2 presents an epidemiological characterization of the 68 CHIKV-confirmed deaths analyzed in this study. CHIKV deaths occurred predominantly in adults aged ≥40 years (n = 42/68 [61.8%]), with 29.4% (20/68) in middle-aged adults and 32.4% (22/68) in the elderly. The average age was 47.6 years (median, 51 years), ranging from 3 days to 85 years. Most CHIKV deaths occurred in females (n = 37/68 [54.4%]). We also report 5 CHIKV deaths in infants and 5 in children (Figure 2A).

The general clinical manifestations presented by CHIKV deaths were fever (n = 44/68 [64.7%]), arthralgia (n = 29/67 [43.3%]), cardiac symptoms such as cardiac arrest (n = 23/67 [34.3%]), dyspnea (n = 23/68 [33.8%]), diarrhea (n = 23/68 [33.8%]), neurological symptoms such as confusion and syncope (n = 17/67 [25.4%]), headache (n = 13/68 [19.1%]), and renal failure (n = 4/68 [5.9%]) (Figure 2B). The average time between the onset of symptoms and death was 12 days (range, 1–90 days). Out of the 68 CHIKV deaths, 79.4% (54/68) were patients with acute infection with fatality occurring up to 18 days from the onset of symptoms. On the other hand, 16.2% (11/68) of fatal cases were patients with subacute infection. It was not possible to obtain information on the date of symptom onset for 3 patients (4.4%). Of the 36 fatal cases with CHIKV RNA positive in CSF, 3 CHIKV deaths were patients presenting with up to 20 days of infection.

Subsequently, we analyzed comorbidity and immunosuppression records available for 65% (44/68) of CHIKV deaths. No comorbidities were reported in 45.5% (20/44) of the medical records, while 27.3% (12/44) had 1 comorbidity, 25% (11/44) had 2 comorbidities, and only 2.3% (1/44) had 3 comorbidities. The most frequent comorbidities were hypertension in 40.9% (18/44) and diabetes in 15.9% (7/44) of CHIKV deaths (Figure 2D). All patients with diabetes also had hypertension, but only 38.9% (7/18) of patients with hypertension had diabetes.

The average age of CHIKV deaths in people with comorbidities (60 years [range, 31–85 years]) differs from the average age of CHIKV deaths in those without comorbidities (37.4 years [range, 3 days–79 years]) (P = .003, Mann-Whitney U test). Multivariable logistic regression analysis, controlled for age and sex, suggests that the risk of dying during the subacute phase of CHIKV infection increases 7 times in cases with diabetes when compared to cases without diabetes (odds ratio, 7.7; P = .033; Table 1 and Supplementary Table 1). No statistically significant difference was observed between the days to death of patients with and without comorbidities (P = .2855, Mann-Whitney U test). No immunosuppression by cancer, human immunodeficiency virus, or corticosteroid treatment was reported in CHIKV deaths.

For 42 (61.8%) autopsied CHIKV deaths, heart and/or respiratory failure were the most frequent causes of death (76.2% [32/42]). Autopsies revealed vascular congestion and edema in main organs

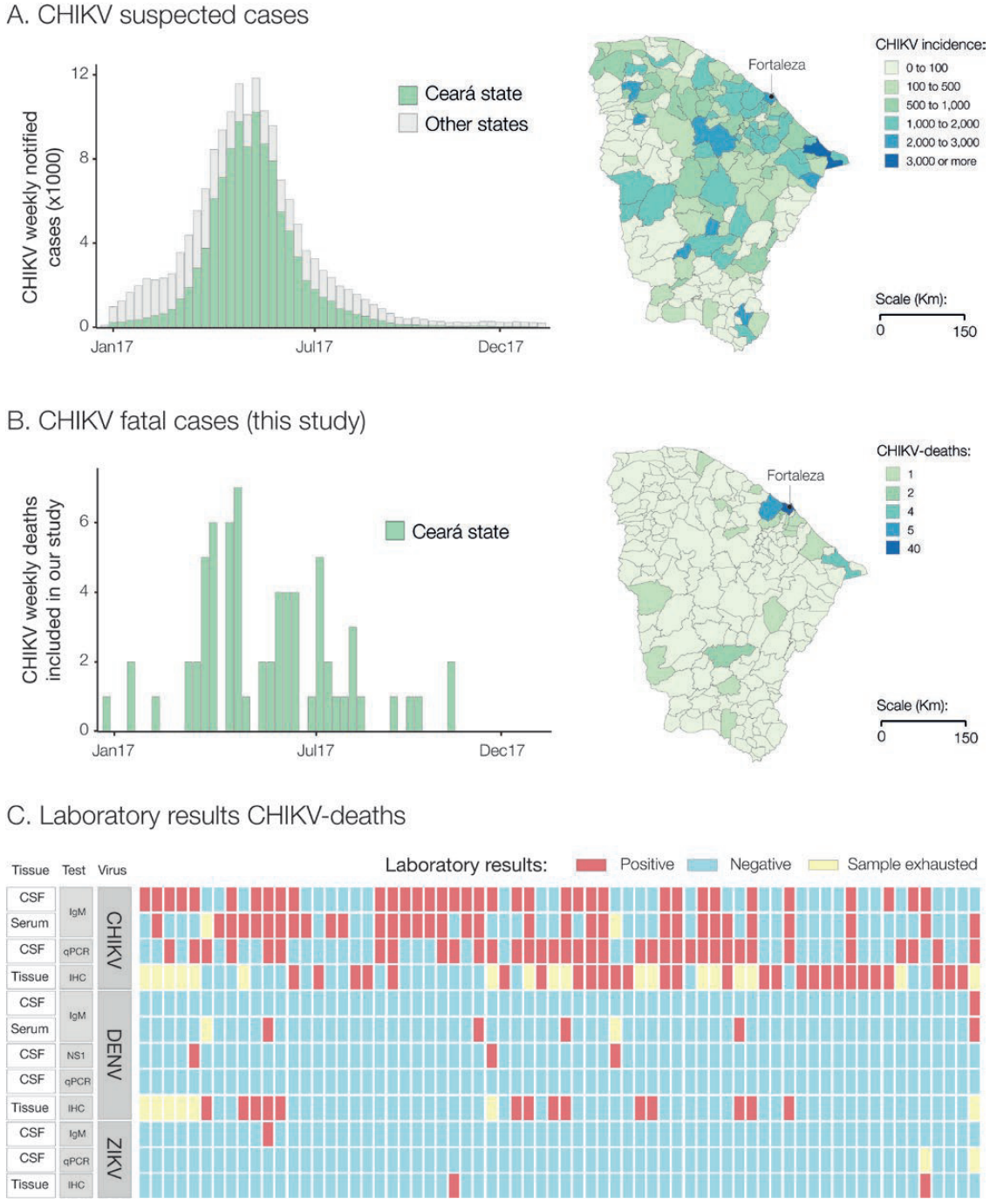

Figure 1. *A*, Weekly epidemiologic curve of chikungunya cases notified in Ceará state and other Brazilian states, and incidence of chikungunya cases notified by municipalities in Ceará state in 2017. *B*, Weekly epidemiologic curve and geographical distribution of chikungunya deaths described in this study. *C*, Diagnosis of 68 Chikungunya deaths described in this study. Abbreviations: CHIKV, chikungunya virus; CSF, cerebrospinal fluid; DENV, dengue virus; IgM, immunoglobulin M; IHC, immunohistochemistry; qPCR, quantitative polymerase chain reaction; ZIKV, Zika virus.

of CHIKV deaths (Figure 3). Also, CHIKV deaths were frequently associated with hepatitis (58.5% [24/41]), pneumonitis (52.4% [22/42]), myocarditis (36.6% [15/41]), and encephalitis (21.4% [9/42]) (Figure 3 and Supplementary Table 2). Other frequent findings in the lungs were hemorrhage in 57.1% (24/42), 54.8% (23/42) atelectasis, 33.3% (14/42) megakaryocytes, and 26.2% (11/42) hemosiderophages (Supplementary Figure 1).

To elucidate the genetic diversity of CHIKV strains in Ceará, we sequenced the viral genomes from 7 samples recovered from 6 CHIKV deaths (patients 8, 12, 22, and 27 from CSF samples; patient 59 from blood; and patient 4 from CSF and blood). ML and Bayesian phylogenies suggested that sequenced CHIKV strains formed 2 monophyletic clades (1 and 2) with maximum statistical support within the ECSA lineage circulating in Brazil (bootstrap score = 100, posterior

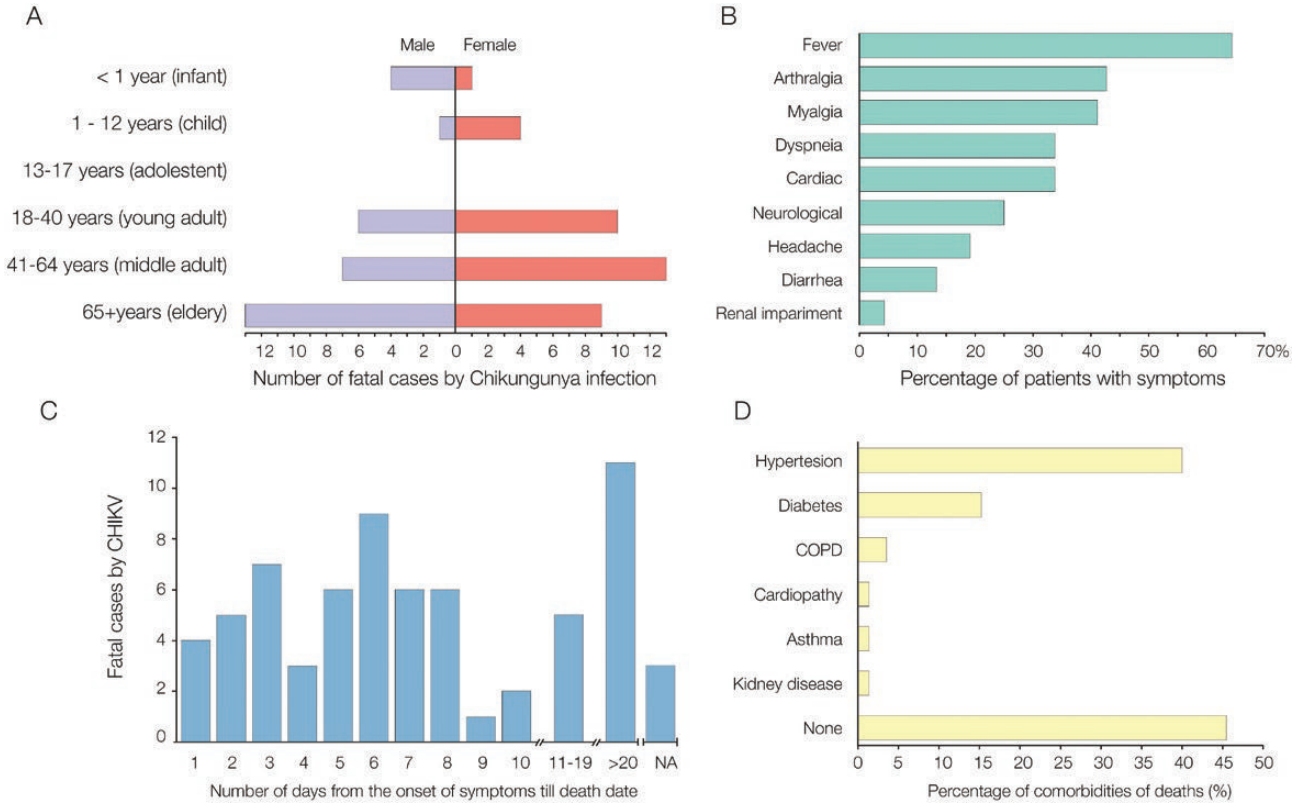

Figure 2. Demographics, symptoms, and comorbidities of 68 chikungunya deaths from Ceará state, Brazil. *A*, Age range and sex. *B*, Clinical characteristics. *C*, Days from the onset of symptoms of individuals till death. *D*, Comorbidities associated with chikungunya deaths. Abbreviations: CHIKV, chikungunya virus; COPD, chronic obstructive pulmonary disease; NA, not available.

support = 1.00). Our analyses suggest that CHIKV was introduced into Ceará state between early 2015 and mid 2016, with the most common ancestor for each cluster at around early to mid-2016 (cluster 1: 2016.60 [95% highest posterior density {HPD}, 2016.39–2016.90]; cluster 2 = 2016.84 [95% HPD, 2015.5–2016.83]) (Figure 4). Based on the analysis of the genomes obtained in this study compared to the genomes available, we did not find mutations associated with enhanced infection and transmission in mosquitoes, or increased virulence, and no unique mutations associated with the Ceará sequences.

DISCUSSION

Herein, we perform the most comprehensive characterization with description of clinical, demographic, and laboratory findings of the largest cross-sectional study population of confirmed CHIKV deaths to date. We confirm CHIKV infection in 68 cases and exclude co-detection with ZIKV and/or DENV in 73.5% of them. Although comorbidities and older age play an important role in CHIKV deaths, we show that almost half of fatal cases did not have any comorbidities and that 38.2% (26/68) of them were

Table 1. Univariate and Multivariable Logistic Regression Analysis of the Presence of Acute or Subacute Fatalities by Chikungunya Infection

	Risk Fatality During Subacute Disease					
Symptoms	Unadjusted OR	(95% CI)	*P* Value	φ Adjusted OR	(95% CI)	*P* Value
Hypertension						
No	Ref	Ref	Ref	Ref	Ref	Ref
Yes	2.77	(.58–13.2)	.200	4.1	(.62–26.9)	.141
Diabetes						
No	Ref	Ref	Ref	Ref	Ref	Ref
Yes	6.93	(1.2–40.9)	**.033**	**7.7**	(1.2–50.0)	**.033**
Diabetes and hypertension						
No	Ref	Ref	Ref	Ref	Ref	Ref
Yes	1.50	(.20–10.8)	.687	2.48	(.20–29.4)	.472

φ adjusts by sex (OR, 0.96; *P* = .966) and age (OR, 1.00; *P* = 9.66).

Abbreviations: CI, confidence interval; OR, odds ratio. Results with $p < 0.05$ are shown in the bold.

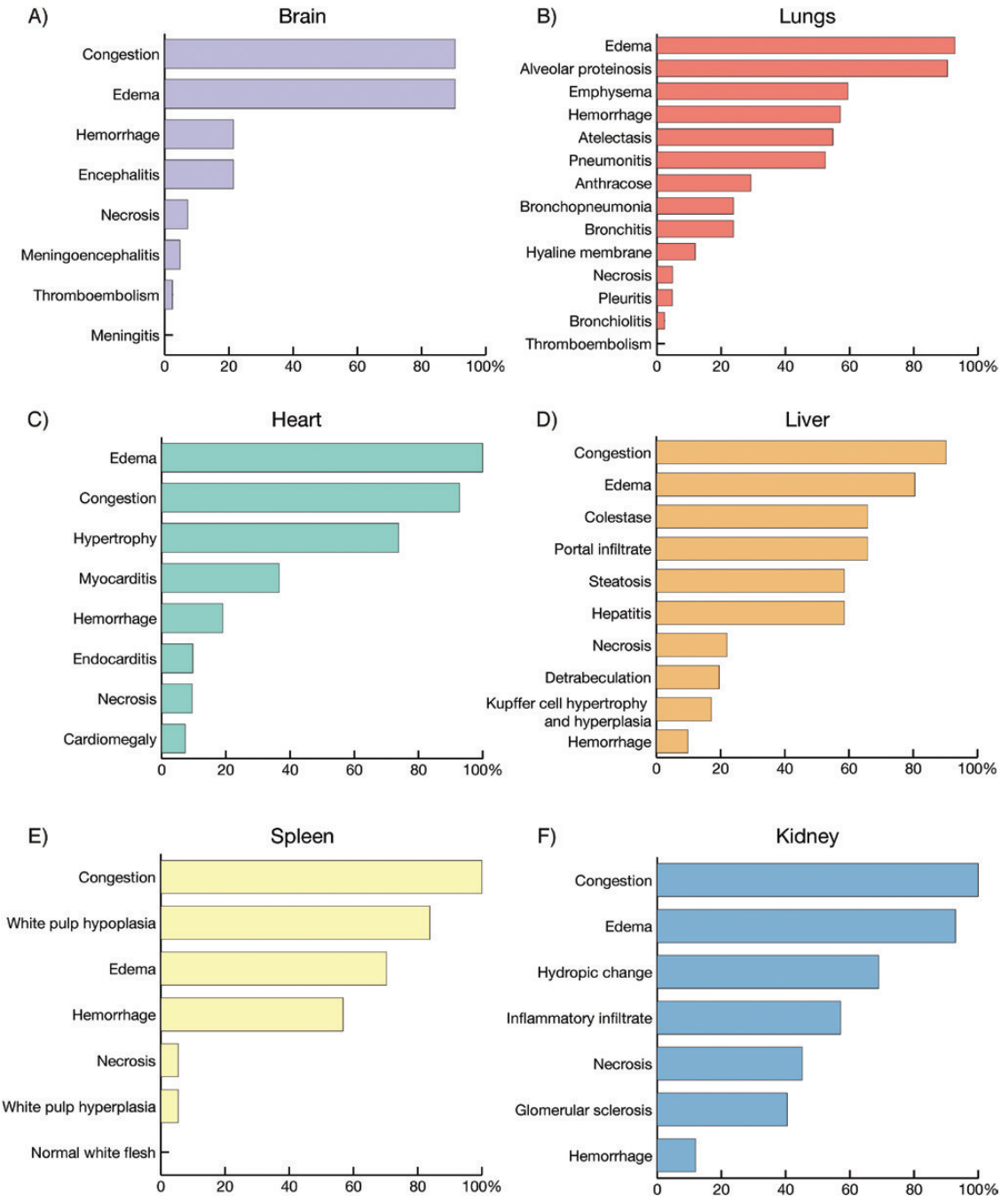

Figure 3. *A–F*, Autopsy findings of 42 chikungunya deaths from Ceará state, Brazil.

aged ≤40 years. In addition, our autopsy results point to cardiac and respiratory failure, possibly due to generalized congestion and edema as the main death cause in the course of CHIKV infection.

In contrast to other arboviral diseases (eg, DENV and ZIKV), CHIKV infection is symptomatic in most individuals, manifesting as a typical rapid-onset febrile disease, characterized by intense arthralgia, myalgia, headache, and rash [2]. The clinical manifestations described herein for the 68 CHIKV deaths were consistent with previous case reports of typical CHIKV infections [2]. However, the detection of CHIKV RNA in the CSF of 36 patients and 4 brain samples and the high frequency of neurological symptoms are strongly indicative of a neurotropic role of CHIKV associated with a severe central nervous system infection in more than half of CHIKV-deaths.

CHIKV deaths have only been reported as an outcome of acute infection [15–20]. However, 16.2% of the fatal cases in our study were subacute CHIKV infection, with symptom onset within 25–90 days prior to death, showing that subacute

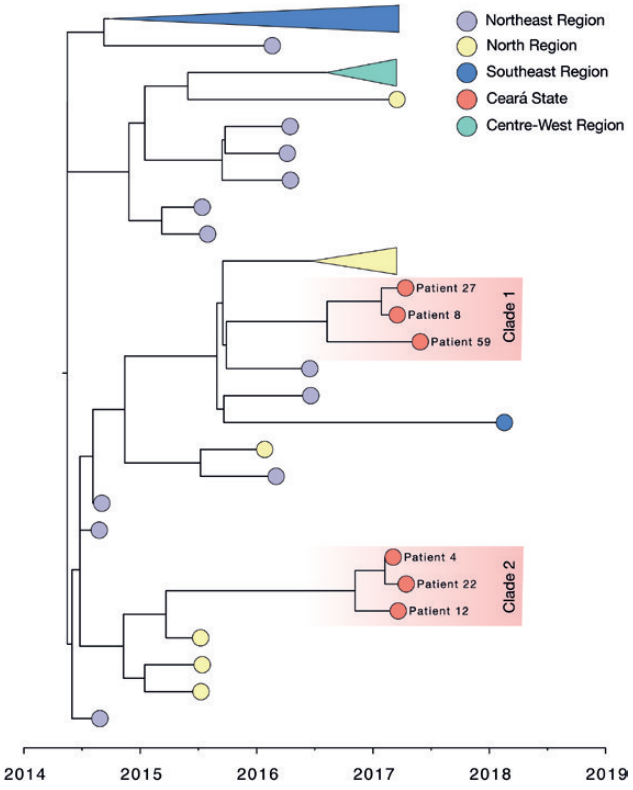

Figure 4. Maximum clade credibility tree of the East-Central-South African genotype of Chikungunya virus in Brazil (n = 71), including 6 new sequences from Ceará State. Tips are colored according to the source region of each sample. Clusters from the Southeast, Center-West, and North regions have been collapsed for better visualization. The 2 clusters of sequences from this study were identified as clade 1 and clade 2 based on the earliest estimated time to most recent common ancestor. A molecular clock approach was used for generating the time-rooted tree (see Methods).

CHIKV infections may also have a fatal outcome. These findings are consistent with studies that demonstrate that the peak of excess deaths occurs with a lag of 1 month in relation to reported cases of chikungunya [33]. Subacute CHIKV deaths especially may be underreported as their long-term duration might decrease the idea of an association between a CHIKV diagnosis and a deadly outcome, while increasing the apparent importance of comorbidities or even hospital-acquired infections as the main cause of death [6].

Severe chikungunya infection has been correlated with age dependency and follows a U-shaped parabolic curve [2]. Here, we confirmed that middle-aged adults and the elderly constituted the majority of CHIKV deaths, followed by infants and children, while no fatal cases in adolescents were identified [2]. However, 23.5% of CHIKV deaths occurred in young adults, indicating that deaths by CHIKV infection do occur at young ages more than previously anticipated. Future clinical and epidemiological studies of CHIKV could help to shed light on the risk of infection and disease severity per age class.

Previously reported risk groups for severe chikungunya infection were patients with comorbidities or those immunocompromised [2]. Our results partially support this conclusion, as 54.5% of our cases had at least 1 comorbidity. Notably, we observed that diabetes considerably increases the risk for death in the subacute phase of CHIKV infection. However, 45.5% did not have any comorbidity reported in their medical records. In addition, none of our cases had any medical history of immunosuppression. Collectively, these results suggest that chikungunya infection can lead to patient death even in the absence of an underlying medical condition.

Our autopsy and histopathological analyses suggest that multiple organ dysfunction syndrome in CHIKV infection may occur by hemodynamic disturbance (vascular congestion, edema, and hemorrhage) of main organs, predominately heart and lungs. Also, the presence of hemosiderophages and megakaryocytes within the lungs suggests a role for increasing pressure on alveolar capillaries leading to hemorrhagic complications, as previously described for DENV [34, 35]. Therefore, careful monitoring of fluid balance and administration of hypotonic solutions can be required as part of clinical management.

CHIKV has widely spread throughout Ceará state. Our genetic analysis revealed that virus strains circulating in the state belong to the CHIKV-ECSA genotype introduced in Bahia state in 2014 [4]. We found no amino acid mutations associated with enhanced infection and transmission in mosquito vectors [11].

Our results demonstrated that CHIKV-associated deaths are not a rare event during large outbreaks, and may occur even in low-risk populations (young age and no comorbidities). Thus, the total disease burden must be reevaluated considering these outcomes. Guidelines and diagnoses need to be improved to prevent fatal outcomes in CHIKV-infected patients.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. S. T. S. L., L. M. S. M., F. M. C. A., I. L. C. R., M. J. M. S., C. P. S. L., R. R. B., and M. P. C. S. performed the RT-qPCR and serological tests. F. K. A. B., D. N. M. B., A. R. S., R. M. A. B. O., C. S. L., M. N. P. M., L. M. F., F. R. F. T., D. R. Q. L., and C. H. M. A. performed the histopathological and immunohistochemistry analysis. W. M. S., D. S. C., and M. J. F. performed viral genome sequencing. R. J., V. S. F., L. H. D., A. L. E. A., and E. L. L. A. provided the epidemiological data. D. S. C. and N. R. F. performed the bioinformatics work and assisted with phylogenetic analyses. D. S. C., J. P. C., and N. R. F. performed the statistical analyses. A. R. R. F., J. L. S. G. V., L. T. M. F., L. P. G. C., and F. M. oversaw parts of the laboratory work. J. P. C., O. G. P., L. T. M. F., M. R. T. N., L. P. G. C., and F. M. contributed to the overall design, interpreted results, and commented on article drafts. W. M. S., D. S. C., M. J. F., S. T. S. L., and N. R. F. wrote the article. W. M. S. led the design and execution of the study and oversaw all analyses and interpretation. All authors have seen and approved the final submitted article.

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Supplementary Appendix

Supplementary Table 1. Demographic and comorbidities of CHIKV fatal cases (n=68)

Characteristics		N (%)
Sex		
	Female	38 (44.1)
	Male	30 (55.9)
Age*		47.7 ± 24.5
Stage of disease at death (n=68)		
	Acute (0-20 days)	54 (79.4)
	Sub-acute (>20 days)	11 (16.2)
	Without information	3 (4.4)
Hypertension (n=41)		
	Yes	19 (46.3)
	No	22 (53.7)
Heart disease (n=39)		
	Yes	1 (2.6)
	No	32 (97.4)
DOPC (n=39)		
	Yes	2 (5.1)
	No	37 (94.9)
Asthma	(n=38)	
	Yes	1 (2.6)
	No	37 (97.4
Kidney failure (n=39)		
	Yes	1 (2.6)
	No	38 (97.4)
Hypertension and diabetes		
	Yes	10 (31.25)
	No	22 (68.8)

* Mean ± standard deviation

Supplementary Appendix

Supplementary Table 2. Autopsy findings of 42 chikungunya deaths from Ceará state, Brazil.

Brain			
Findings	**Absent**	**Discreet**	**Moderate/ Intense**
Congestion	9.5% (4/42)	47.6% (20/42)	42.9% (18/42)
Edema	9.5% (4/42)	47.6% (20/42)	42.9% (18/42)
Hemorrhage	78.6% (33/42)	19.0% (8/42)	2.4% (1/42)
Meningitis	100% (42/42)	0% (0/42)	0% (0/42)
Meningoencephalitis	95.2% (40/42)	2.4% (1/42)	2.4% (1/42)
Necrosis	92.8% (39/42)	2.4% (1/42)	4.8% (2/42)
Thromboembolism	97.6% (41/42)	0% (0/42)	2.4% (1/42)
Encephalitis	78.6% (33/42)	19.0% (8/42)	2.4% (1/42)
Lung			
Findings	**Absent**	**Discreet**	**Moderate/ Intense**
Atelectasis	45.2% (19/42)	50.0% (21/42)	4.8% (2/42)
Anthracose	70.7% (29/41)	29.3% (12/41)	0% (0/41)
Bronchopneumonia	76.2% (32/42)	4.8% (2/42)	19.0% (8/42)
Bronchitis	76.2% (32/42)	19.0% (8/42)	4.8% (2/42)
Bronchiolitis	97.6% (41/42)	2.4% (1/42)	0% (0/42)
Alveolar proteinosis	9.5% (4/42)	11.9% (5/42)	78.6% (33/42)
Edema	7.2% (3/42)	45.2% (19/42)	47.6% (20/42)
Emphysema	40.5% (17/42)	50% (21/42)	9.5% (4/42)
Hemorrhage	42.9% (18/42)	42.9% (18/42)	14.2% (6/42)
Pneumonitis	47.6% (20/42)	40.5% (17/42)	11.9% (5/42)
Hyaline Membrane	88.1% (37/42)	7.1% (3/42)	4.8% (2/42)
Necrosis	95.1% (39/41)	4.9% (2/41)	0% (0/41)
Pleuritis	95.1% (39/41)	2.4% (1/41)	2.4% (1/41)
Thromboembolism	100% (42/42)	0% (0/42)	0% (0/42)
Heart			
Findings	**Absent**	**Discreet**	**Moderate/ Intense**
Congestion	7.1% (3/42)	71.4% (30/42)	21.4% (9/42)
Hemorrhage	81.0% (34/42)	19.0% (8/42)	0% (0/42)
Endocarditis	90.2% (37/42)	7.3% (3/41)	2.4% (1/41)
Edema	0% (0/42)	61.9% (26/42)	38.1% (16/42)
Hypertrophy	26.2% (11/42)	52.4% (22/42)	21.4% (9/42)
Necrosis	90.5% (38/42)	2.4% (1/42)	7.1% (3/42)
Myocarditis	63.4% (26/41)	24.4% (10/41)	12.2% (5/41)
Cardiomegaly	92.7% (38/41)	4.9% (2/41)	2.4% (1/41)
Liver			
Findings	**Absent**	**Discreet**	**Moderate/ Intense**
Colestase	34.2% (14/41)	46.3% (19/41)	19.5% (8/41)
Congestion	9.8% (4/41)	34.1% (14/41)	56.1% (23/41)
Detrabeculation	80.5% (33/41)	12.2% (5/41)	7.3% (3/41)
Edema	19.5% (8/41)	46.3% (19/41)	34.2% (14/41)
Steatosis	41.5% (17/41)	36.6% (15/41)	21.9% (9/41)
Hemorrhage	90.2% (37/41)	9.8% (4/41)	0% (0/41)
Portal infiltrate	34.2% (14/41)	46.3% (19/41)	19.5% (8/41)
Necrosis	78.0% (32/41)	17.1% (7/41)	4.9% (2/41)
Hepatitis	41.5% (17/41)	46.3% (19/41)	12.2% (5/41)
Kupffer cell	82.9% (34/41)	17.1% (7/41)	0% (0/41)

hypertrophy and hyperplasia			
Spleen			
Findings	**Absent**	**Discreet**	**Moderate/ Intense**
Congestion	0% (0/37)	32.4% (12/37)	67.6% (25/37)
Edema	29.7% (11/37)	56.8% (21/37)	13.5% (5/37)
Hemorrhage	43.2% (16/37)	48.7% (18/37)	8.1% (3/37)
Necrosis	94.6% (35/37)	5.4% (2/37)	0% (0/37)
Normal white flesh	100% (37/37)	0% (0/37)	0% (0/37)
White pulp hypoplasia	16.2% (6/37)	51.4% (19/37)	32.4% (12/37)
White pulp hyperplasia	94.6% (35/37)	5.4% (2/37)	0% (0/37)
Kidney			
Findings	**Absent**	**Discreet**	**Moderate/ Intense**
Congestion	0% (0/42)	23.8% (10/42)	76.2% (32/42)
Edema	7.1% (3/42)	42.9% (18/42)	50% (21/42)
Hemorrhage	88.1% (37/42)	11.9% (5/42)	0% (0/42)
Necrosis	54.8% (23/42)	23.8% (10/42)	21.4% (9/42)
Hydropic change	31% (13/42)	57.1% (24/42)	11.9% (5/42)
Inflammatory infiltrate	42.9% (18/42)	38.1% (16/42)	19.0% (8/42)
Glomerular sclerosis	59.5% (25/42)	26.2% (11/42)	14.3% (6/42)

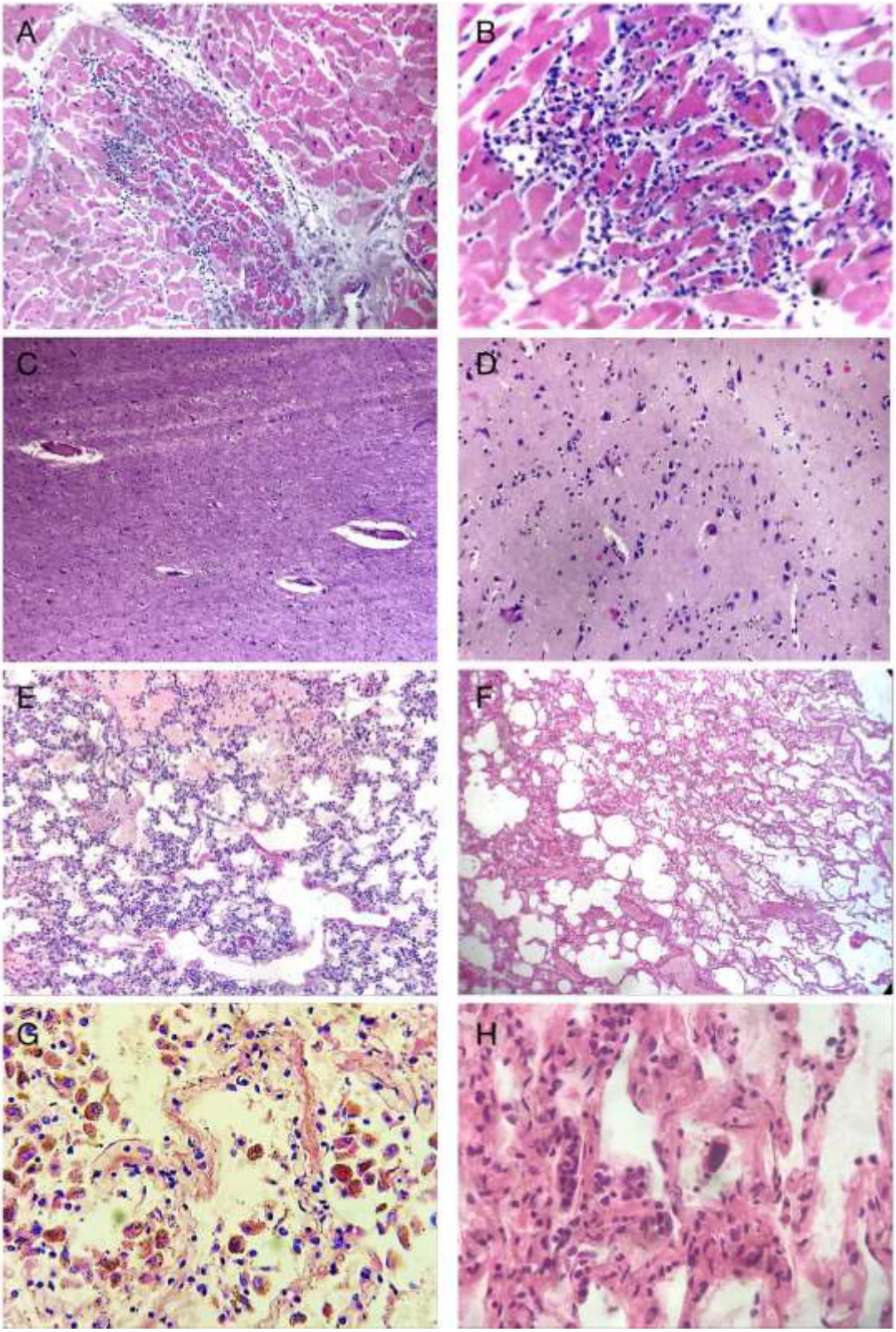

Figure Supplementary 1. Histopathological of chikungunya-deaths in Ceará State. Myocarditis. Mature lymphoplasmacytic infiltrate in cardiac tissue, associated with myocytic necrosis, edema, and neovasal formation. Note myocytes with hypereosinophilia characterizing cellular necrosis (A and B). Encephalitis - Lymphocytic infiltrate in cerebral cortical tissue, associated with reactional gliosis and edema (C and D). Acute bronchopneumonia. Diffuse interstitial infiltrate expanding alveolar septa, with areas of peribronchiolar and perivascular contentment. In some areas, acute interstitial pneumonia pattern and in organization with hyaline membranes (E and F) is observed. (G) Presence of hemosiderophages in lungs of a CHIKV-death. (H) Presence of megakaryocyte in lungs of a CHIKV-death.

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COVID-19 Home Deaths without Medical Assistance in Northeastern Brazil

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Abstract. Since its beginning in Wuhan, China, in December 2019, the disease caused by COVID-19 has reached more than 27 million confirmed cases and more than 880 thousand deaths worldwide by early September 2020. Although it is known that some of these deaths may have been influenced by the overload of health systems, the world medical literature lacks data on deaths due to COVID-19 in patients who have not received medical assistance. We conducted a retrospective transversal study to report the clinical and epidemiological profile of the first 200 consecutive cases of home deaths without medical assistance caused by COVID-19 diagnosed by verbal autopsy and real-time PCR in samples of postmortem nasopharyngeal swabs, in the state of Ceara, in Northeastern Brazil. The data show a slightly increased prevalence of cases in males (57%) and an average age of 76.8 years. Previous comorbidities were reported in 85.5% of cases, the most common being cardiovascular disease (45%), neurological disease (30%), and diabetes (29%). The main symptoms reported were dyspnea (79%), fever (75.5%), cough (69%), and fatigue (42.5%). The average time between the onset of illness and death was 7.3 days, being statistically shorter in patients who had previous comorbidities ($P = 0.0215$). This is the first study to evidence the clinical and epidemiological characteristics of COVID-19 home deaths without medical assistance, which may represent a considerable portion of the pandemic burden, especially in the context of health system overload.

INTRODUCTION

Since its beginning in Wuhan, China, in December 2019, the disease caused by COVID-19 has reached more than 200 countries, and, as of early September 2020, there were more than 27 million confirmed cases and 880 thousand deaths from COVID-19 worldwide, from which four million confirmed cases and 126 thousand deaths occurred only in Brazil.[1]

The most lethal clinical manifestation of COVID-19 is the severe acute respiratory syndrome,[2] which demands hospitalization and intensive care treatment. Thus, the rapid spread of the disease has led to the overload of health systems, that is, the inability to provide health care in response to growing demand, which occurred even in developed countries such as the Italian region of Lombardy[3] and the Spanish autonomous region of Madrid,[4] contributing to the rise in mortality for the insufficient or even the absence of health assistance during the disease.

In the state of Ceara, in Northeastern Brazil, the health system has overloaded only 45 days after the first notified case of COVID-19,[5] leading to the rise of deaths without medical assistance. Most of these home deaths were investigated by the "Dr. Rocha Furtado" Death Verification Service (SVO-RF) located in the state capital city of Fortaleza, which allowed the execution of this retrospective study to describe the clinical and epidemiological characteristics of patients who died of COVID-19 in their homes, without medical assistance.

Surprisingly, to the best of our knowledge, there are no reports referring to deaths by COVID-19 that lacked medical assistance to this date, underscoring the relevance of this report.

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METHODS

Participants. We retrospectively enrolled the first 200 consecutive home deaths by COVID-19 assessed by the SVO-RF, which occurred between March 25 and May 12, 2020, in the state of Ceara, in northeastern Brazil. These patients were all domiciled in the state capital city of Fortaleza and its metropolitan area. The period corresponds to the first phase of the pandemic in the state of Ceara—which peaked in the 19th epidemiological week (from May 3 to 9) and started to decrease from the 20th epidemiological week (from May 10 to 16)—and was chosen for being the most critical period for the health systems when the characteristically exponential growth was presented.[6]

Deaths without medical assistance. Deaths that occurred at home, but have received significant medical assistance during the disease that led to death, were not assessed by the SVO-RF, and therefore were not included in this study. For example, patients assisted by family, geriatric, or palliative physicians, when dying at home, have their deaths assessed by their medical doctors. Patients who did not have regular medical assistance but sought emergency assistance during their last hours of life and died in the emergency room had their deaths assessed by the emergency physician, and were also not included in this study. For the same reasons, deaths at nursing homes were not included. Therefore, patients included in this study have not received any medical assistance at all or have received some limited medical assistance like emergency consultations, but not during the dying process.

Assessment of death by COVID-19. During the COVID-19 pandemic, the SVO-RF followed the Brazilian Ministry of Health's guideline[7] to suspend complete diagnostic autopsies in confirmed or suspected cases of COVID-19. Thus, during the pandemic, SVO-RF assessed the causes of deaths using verbal autopsy and, in selected cases, collecting noninvasive biological samples. The SVO-RF verbal autopsy protocol consists of an interview with the closest relatives regarding previously known comorbidities, signs, symptoms, and chronological

sequence of events that lead to death, as most of the verbal autopsy protocols, but it differs from them for having experienced physicians not only to validate the cause of death but also to execute the interview and the external physical examination of the body,[8] which improves the completeness of the medical records.

Cases were considered suspected for COVID-19 if the deceased presented flu-like symptoms or had close contact with another suspected or confirmed case of COVID-19. For suspected cases, postmortem nasopharyngeal swabs were collected and sent to the Central Laboratory of Public Health of Ceara for real-time PCR study, based on the Charité protocol. Standard laboratory protocols and complete individual protection equipment were used for both workers' safety and for avoiding contamination of the samples, procedures that were already performed as part of the routine of SVO-RF. If the study resulted in the detection of SARS-CoV-2 genetic material, the case was considered a confirmed death due to COVID-19.

Data analysis. The SVO-RF medical records were reviewed for socio-epidemiological (gender, age, race, education level, occupation, and marital status) and clinical variables (symptoms, comorbidities, and time from illness onset to death). Estimation of any health measures during the disease, including self-medication or alternative medicines, was attempted, but the information was most often incomplete or conflicting because of the lack of medical records and because of the poor socio-educational level of the family of the deceased. Therefore, this variable could not be analyzed.

The clinical and epidemiological characteristics between genders were compared with the Student's *t* and chi-square tests. The variable "time from illness onset to death" was analyzed as a clinical outcome, and its correlations with other epidemiological and clinical characteristics were assessed with Pearson's linear regression and Student's *t*-tests. *P*-value lower than 0.05 was considered statistically significant.

Ethics. This study was approved by the Research Ethics Office of the School of Public Health of Ceara (approval number: 33464720.0.0000.5037) and followed the international ethics guidelines for human research rigorously.

RESULTS

The epidemiological and clinical characteristics of all 200 patients who died due to COVID-19 at home, without medical assistance, are summarized in Table 1.

Of these, 114 patients (57%) were male and 86 (43%) were female. The mean age of the patients was 76.82 ± 14.94 years, and the range of age varied from 25 to 101 years. The distribution of cases by age showed a peak between 70 and 79 years, with 64 cases (32.0%). The age of deceased male patients (74.53 ± 14.82 years) was statistically younger than females (79.85 ± 14.65 years; $P = 0.0123$).

The distribution of cases by race showed a prevalence of cases in browns (145 patients, 72.5%), followed by white (50 patients, 25%) and black (five patients, 2.5%). As for the education level, 56 patients (28.0%) had no formal education, 107 (53.5%) had only elementary education, 29 (14.5%) had completed high school, and only five (2.5%) had completed a college degree. Three cases (1.5%) had their education levels ignored by family. There was no statistical difference between males and females regarding race or education level.

The occupation status showed a high amount of different professions, with more than 56 listed. Only two occupations were cited more than 12 times: housewives and farmers, both presenting statistical differences between genders. All the 49 housewives were females and represented 57.0% of all deceased women in this study. There were 24 farmers, with 18 of them being males and six females. Farmers were associated with a statistically shorter period from illness onset to death (see in the following text).

When comparing marital status and genders, there was a statistically higher prevalence in widow among women (45.3%) and married among men (44.7%), without a difference in the proportion of single and divorced patients.

Table 1 also shows the clinical profile of the 200 patients. The main presenting symptoms were dyspnea, reported in 158 patients (79%); fever, reported in 151 (75.5%); cough, reported in 138 (69.0%); and adynamia, reported in 85 patients (42.5%).

Of the total number of cases, 171 patients (85.5%) had at least one comorbidity, whereas 29 patients (14.5%) had no comorbidity. The most common comorbidities were cardiovascular diseases, present in 90 cases (45.0%), followed by neurological diseases in 60 cases (30.0%), diabetes in 58 cases (29.0%), respiratory diseases in 18 cases (9.0%), psychiatric conditions in 11 cases (5.5%), neoplastic diseases in nine cases (4.5%), kidney diseases in eight cases (4.0%), and other less frequent comorbidities were reported in 39 cases (19.5%). Chronic smoking was reported in 31 cases (15.5%) and chronic alcoholism in 23 (11.5%).

The mean number of days from onset of symptoms to death was 7.27 ± 5.18 days, with the range varying from 0 to 32 days, and a median of 6 days. There was no statistical difference between genders regarding symptoms, comorbidities, and time from illness onset to death.

Considering "time from illness onset to death" as our clinical outcome, its correlations with the other clinical and epidemiological variables were assessed and summarized in Table 2. Variables with small frequencies were excluded from this analysis because they did not fulfill parametric requirements for the Student's *t*-test.

There was no statistical significance in the association between the clinical outcome and age, gender, race, education level, or marital status. However, our data showed a slightly shorter period from onset to death in female, white, widowed, and people with no formal education.

Regarding occupation, there was a statistically significant shorter period from illness onset to death among farmers, with 5.17 ± 4.17 days, comparing with 7.56 ± 5.25 days among non-farmers ($P = 0.0338$).

There was no statistical significance when comparing the clinical outcome with the presence of each of the major symptoms, and the presence of each of the major comorbidities. However, when comparing the group of deceased people with some comorbidity versus the group without any comorbidity, a statistically significant difference is seen, with the mean days from illness onset to death of 6.92 ± 4.98 days in deceased people with comorbidities and 9.31 ± 5.94 days in people without comorbidities ($P = 0.0215$).

DISCUSSION

"Time from illness onset to death" is an important clinical outcome, once a shorter evolution to death implies a more

TABLE 1
Clinical–epidemiologic profile of 200 home deaths by COVID-19 (state of Ceará, Brazil, March 25, 2020–May 12, 2020)

	Total (n = 200)		Female (n = 86)		Male (n = 114)		
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	P-value*
Age (years)	76.82 ± 14.94	25–101	79.85 ± 14.65	25–101	74.53 ± 14.82	31–100	0.0123†
Race	*n*	%	*n*	%	*n*	%	P-value‡
Browns	145	72.5	61	70.9	84	73.7	0.8811
White	50	25.0	23	26.7	27	23.7	
Black	5	2.5	2	2.3	3	2.6	
Education level	*n*	%	*n*	%	*n*	%	P-value‡
No formal education	56	28.0	29	33.7	27	23.7	0.2615
Elementary school	107	53.5	45	52.3	62	54.4	
High school	29	14.5	9	10.5	20	17.5	
College	5	2.5	1	1.2	4	3.5	
Ignored	3	1.5	2	2.3	1	0.9	
Occupation	*n*	%	*n*	%	*n*	%	P-value‡
Housewives	49	24.5	49†	57.0	0	0.0	< 0.0001†
Farmers	24	12.0	6	7.0	18†	15.8	
Others	127	63.5	31	36.0	96	84.2	
Marital status	*n*	%	*n*	%	*n*	%	P-value‡
Single	54	27.0	29	33.7	25	21.9	0.0002†
Married	67	33.5	16	18.6	51†	44.7	
Widowed	69	34.5	39†	45.3	30	26.3	
Divorced	10	5.0	2	2.3	8	7.0	
Symptoms	*n*	%	*n*	%	*n*	%	P-value‡
Dyspnea	158	79.0	69	80.2	89	78.1	0.8272
Fever	151	75.5	60	69.8	91	79.8	
Cough	138	69.0	55	64.0	83	72.8	
Fatigue	85	42.5	37	43.0	48	42.1	
Runny nose	30	15.0	13	15.1	17	14.9	
Irritability and confusion	30	15.0	18	20.9	12	10.5	
Headache	24	12.0	11	12.8	13	11.4	
Odynophagia	22	11.0	12	14.0	10	8.8	
Diarrhea	19	9.5	9	10.5	10	8.8	
Myalgia	17	8.5	8	9.3	9	7.9	
Hyporexia	10	5.0	4	4.7	6	5.3	
Nausea and vomiting	10	5.0	6	7.0	4	3.5	
Nasal congestion	4	2.0	2	2.3	2	1.8	
Anosmia	2	1.0	0	0.0	2	1.8	
Other symptoms	16	8.0	7	8.1	9	7.9	
Comorbidities	*n*	%	*n*	%	*n*	%	P-value‡
Cardiovascular disease	90	45.0	40	46.5	50	43.9	0.1606
Neurological disease	60	30.0	26	30.2	34	29.8	
Diabetes mellitus	58	29.0	33	38.4	25	21.9	
Respiratory disease	18	9.0	6	7.0	12	10.5	
Psychiatric conditions	11	5.5	5	5.8	6	5.3	
Neoplastic disease	9	4.5	5	5.8	4	3.5	
Kidney disease	8	4.0	3	3.5	5	4.4	
Chronic smoking	31	15.5	11	12.8	20	17.5	
Chronic alcoholism	23	11.5	4	4.7	19	16.7	
Other comorbidities	39	19.5	19	22.1	20	17.5	
Without comorbidities	29	14.5	10	11.6	19	16.7	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	P-value*
Days from illness onset to death	7.27 ± 5.18	0–32	7.17 ± 5.21	0–32	7.34 ± 5.18	0–30	0.8215

* Pearson's linear correlation test.
† Statistically significant.
‡ Student's t-test.

severe disease presentation and, therefore, can be compared with severity and mortality rates. Also, in the context of deaths without medical assistance, it is important to know which groups are more vulnerable and should receive priority health assistance.

According to the current literature, COVID-19 severity and mortality rates are higher among older patients.[9–12] In one of the first national death reports, there was a higher prevalence in men (61.1%) and patients older than 70 years (62.9%) among the first 54 fatal cases of COVID-19 in South Korea.[13] In this current study, we found that the average age of the 200 patients was 76.82 years, with a peak incidence between 70 and 79 years, and a male predominance of 57% of cases, gender whom also presented a statistically younger age in the moment of death, when compared with women (P = 0.0123).

The prevalence of races among home deaths analyzed in our study (72.5% of brown, 25.0% of white, and 2.5% of black) was similar to the prevalence of the general population in the state of Ceara (66.2%, 27.2%, and 5.9%, respectively).[14] There was a slightly shorter period of illness onset to death in the white population than in the non-white deceased people with COVID-19, but with no statistical significance. By contrast, recent studies in the United States[15,16] and in Brazil[17] showed a higher risk of

TABLE 2

Association between days from illness onset to death and clinical–epidemiologic characteristics of 200 home deaths by COVID-19 (state of Ceará, Brazil, March 25, 2020–May 12, 2020).

	Days from illness to death		
	Mean ± SD		*P*-value*
Age (years)	7.27 ± 5.18		0.508
Gender	Female	Male	*P*-value†
	7.17 ± 5.21	7.34 ± 5.18	0.8215
Race	White	Nonwhite	*P*-value†
	6.50 ± 4.18	7.53 ± 5.47	0.2261
Education level	Presence	Absence	*P*-value†
No formal education	6.75 ± 5.42	7.47 ± 5.09	0.3777
Elementary school	7.85 ± 5.46	6.60 ± 4.79	0.0894
High school	6.86 ± 3.82	7.34 ± 5.39	0.6479
Occupation	Presence	Absence	*P*-value†
Housewives	7.49 ± 5.88	7.20 ± 4.96	0.7336
Farmers	5.17 ± 4.17	7.56 ± 5.25	0.0338‡
Marital status	Presence	Absence	*P*-value†
Single	7.57 ± 5.71	7.16 ± 4.99	0.6151
Married	7.67 ± 5.19	7.07 ± 5.19	0.4381
Widowed	6.59 ± 4.21	7.67 ± 5.61	0.1815
Symptoms	Presence	Absence	*P*-value†
Dyspnea	7.35 ± 5.34	6.95 ± 4.61	0.6562
Fever	6.94 ± 4.44	8.29 ± 6.97	0.1147
Cough	7.67 ± 5.42	6.37 ± 4.53	0.1003
Fatigue	7.06 ± 4.94	7.43 ± 5.37	0.6216
Runny nose	8.03 ± 5.59	7.14 ± 5.12	0.3830
Irritability and confusion	7.00 ± 4.85	7.32 ± 5.25	0.7579
Headache	8.46 ± 5.44	7.11 ± 5.14	0.2322
Odynophagia	8.23 ± 7.39	7.15 ± 4.86	0.3599
Diarrhea	6.47 ± 2.95	7.35 ± 5.36	0.4829
Myalgia	5.71 ± 3.06	7.42 ± 5.32	0.1941
Other symptoms	7.81 ± 6.09	7.22 ± 5.11	0.6636
Comorbidities	Presence	Absence	*P*-value†
Cardiovascular disease	7.34 ± 5.41	7.21 ± 5.02	0.8548
Neurological disease	6.32 ± 3.87	7.68 ± 5.62	0.0887
Diabetes mellitus	7.36 ± 5.67	7.23 ± 4.99	0.8729
Respiratory disease	7.44 ± 3.38	7.25 ± 5.34	0.8815
Chronic smoking	8.42 ± 5.26	7.06 ± 5.16	0.1799
Chronic alcoholism	8.48 ± 7.45	7.11 ± 4.82	0.2357
Some comorbidity	6.92 ± 4.98	9.31 ± 5.94	0.0215‡

* Pearson's linear correlation test.
† Student's *t*-test.
‡ Statistically significant.

mortality among nonwhite patients with COVID-19 with medical assistance.

The educational level survey demonstrates the vulnerability of the analyzed population, with 28.0% with no formal education and 53.5% with only elementary school, which may have contributed to the inaccessibility of health systems along with the saturation of the health facilities by the pandemic itself.

Our study identified a statistically significant shorter period from illness onset to death among farmers, contrasting with the absence of reports relating farmers with increased risk for mortality by COVID-19. Only a single study[18] evidenced similar occupations, such as animal slaughtering and processing industry, as being classified as essential industries during the pandemics and being more exposed to infections.

Concerning the symptoms of COVID-19, one of the first reports from China[19] evidenced as the most commonly reported symptom fever (71.4%), cough (60.4%), and fatigue (43.9%). A large systematic review and meta-analysis[12] showed similar results: fever (78.5%), cough (53.8%), and fatigue (25.0%). However, another systematic review[20] stated that dyspnea was the most significant symptom associated with lethal disease, and the mode of death was predominantly through respiratory or heart failure, which aligns with our findings that evidenced that the most common symptoms associated with home deaths were dyspnea (79.0%), followed by fever (75.5%), cough (69.0%), and fatigue (42.5%).

Among all patients diagnosed with COVID-19 analyzed in a large systematic review,[12] comorbidities were present in 31.0% of the adult patients, with hypertension being the most prevalent, followed by heart failure, diabetes mellitus, and coronary heart disease, and its meta-analysis also revealed that preexisting comorbidities were associated with a higher relative risk (RR) of disease severity (RR = 2.11 [1.02–4.35]; $P = 0.046$) and in-hospital mortality (RR = 1.69 [1.48–1.94]; $P < 0.001$). In addition, a report focused only on the analysis of deceased patients by COVID-19 showed a much higher prevalence of comorbidities (68.2%), especially hypertension (37.6%), diabetes (22.4%), and coronary heart disease (11.8%).[21] These last numbers are closer to the ones in our study when we focus only in deceased patients who died at home without medical assistance and found that the majority of patients had some previous comorbidity (85.0%), with the most common being cardiovascular diseases (45%), neurological diseases (30%) and diabetes (29.0%). It is reinforced that the presence of previous comorbidities was significantly related to the reduction in the time between illness onset and death in our study.

We conclude that this is a pioneer report not only for evidencing the clinical and epidemiological profile of patients who died because of COVID-19 without medical assistance but also for demonstrating the similarities and particularities against the current known profile of COVID-19 deaths, which are composed exclusively of medical-assisted deaths.

However, there were three major limitations of this study. The first one was the impossibility of conducting a complete diagnostic autopsy (CDA) with a detailed pathological study of the organs. In performing a CDA, one could potentially rule out some of these deaths that might have been caused by other etiologies in patients who were only carriers of the virus and were manifesting mild active disease, and could also allow a better understanding of the pathophysiology of COVID-19.[22–24]

The second limitation is the absence of ancillary examinations and the dependence in oral testimonials of the poor socio-educational level families to collect data for the verbal autopsy, which may have led to the partial missing of some symptoms, comorbidities, and even the non-suspicion of cases with atypical presentation.

The third limitation was the impossibility to address the major reasons for the absence of medical assistance during these COVID-19 home deaths, as this question was not usually asked in the verbal autopsy protocol and was not registered in the medical records. Therefore, subsequent studies are necessary to evaluate quantitatively and qualitatively why these patients did not receive health care before death, with the overload of the health system being our major hypothesis.

Despite its limitations, the death investigation conducted by SVO-RF greatly enhanced the detection of COVID-19 deaths that would certainly be underreported. It also helped understand the clinical and epidemiological characteristics of this vulnerable portion of the society that lacks access to health systems and may represent a considerable portion of the pandemic burden, especially in the context of health system overload.

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PLOS NEGLECTED TROPICAL DISEASES

VIEWPOINTS

Validation of verbal autopsy and nasopharyngeal swab collection for the investigation of deaths at home during the COVID-19 pandemics in Brazil

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Brazil currently holds the second highest record of cases and deaths due to the Coronavirus Disease 2019 (COVID-19) in the world [1]. The state of Ceará, with >9 million inhabitants, in Northeastern Brazil, was the epicenter of the epidemic and one of the first states to present community transmission of the COVID-19. Forty-five days after the first confirmed case, there was a collapse of health services, contributing to the increase in the number of people dying at home [2].

In the scenario of a pandemic associated with an increase in the number of deaths outside a hospital environment, a specialized service in the investigation of fatalities was necessary to increase the capacity of the health system and to detect and report the death burden from the disease. "Dr. Rocha Furtado" Death Verification Service (SVO-RF) is located in the capital Fortaleza and, in partnership with the Epidemiological Surveillance Office and the local public health laboratory, it acts in the investigation of deaths in the state, which was already reported in recent arboviruses epidemics [3,4].

To fulfill its mission, SVO-RF performs complete diagnostic autopsies (CDA) for the investigation of deaths at home without medical assistance, as well as deaths without bona fide diagnosis prior to death [5]. Moreover, unlike most other Death Verification Services in Brazil, the SVO-RF also implemented a medical team that drives to the houses where the death occurred, to investigate cases where a clinical necropsy was not indicated, such as with patients with advanced cancer diagnoses or other chronic terminal illnesses that die at home, without any home care program. This team, called SVO-Mobile, is composed of a physician, a social worker, and a driver and usually acts in a complementary way to clinical necropsies that take place at the headquarters. In the COVID-19 pandemic, however, the SVO-Mobile played a significant role since clinical necropsies were suspended following the Ministry of Health's guidelines [6].

Due to the lack of biosafety at the headquarters, including an air treatment system at the facility, added to the community transmission of the virus and the risk of transmission by asymptomatic and presymptomatic patients, all autopsies were suspended. The SVO-RF leadership saw then the need to expand the number of SVO-Mobile teams from 1 to 3, while still maintaining its regular team at the headquarters. All deaths from this period were investigated

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Table 1. Deaths at home in Fortaleza, State of Ceará, attended by SVO-RF in 2018, 2019, and 2020, suspicious and confirmed home deaths by COVID-19 in 2020.

EW	N of home deaths in 2018	N of home deaths in 2019	N of home deaths in 2020	% change in the number of deaths (2020/average 2018–2019)	N of suspected COVID-19 deaths in 2020	% of suspected COVID-19 deaths in 2020	N of deaths by COVID-19 confirmed in 2020	% of deaths by COVID-19 confirmed in relation to suspects in 2020
10	63	56	58	−2.52	0	0.00	0	0.00
11	59	66	57	−8.80	0	0.00	0	0.00
12	62	64	62	−1.59	1	1.61	0	0.00
13	64	66	76	16.92	4	5.26	2	50.00
14	60	49	75	37.61	5	6.67	1	20.00
15	53	66	82	37.82	9	10.98	5	55.56
16	53	52	103	96.19	14	13.59	7	50.00
17	72	70	96	35.21	15	15.63	13	86.67
18	59	60	169	184.03	39	23.08	39	100.00
19	46	69	262	355.65	86	32.82	78	90.70
20	77	66	236	230.07	89	37.71	75	84.27
21	55	66	173	185.95	41	23.70	37	90.24
22	47	45	118	156.52	20	16.95	15	75.00
23	62	53	85	47.83	8	9.41	3	37.50
24	38	53	75	64.84	6	8.00	2	33.33
25	42	48	64	42.22	3	4.69	2	66.67
26	56	60	50	−13.79	4	8.00	1	25.00
27	62	43	57	8.57	3	5.26	0	0.00
28	68	49	58	−0.85	1	1.72	0	0.00
29	60	46	63	18.87	2	3.17	0	0.00
30	58	45	44	−14.56	2	4.55	0	0.00
31	52	43	52	9.47	1	1.92	0	0.00
Total	**1,268**	**1,235**	**2,115**	**69.00**	**353**	**16.69**	**280**	**79.32**

EW, epidemiological week; N, number.

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through physician-certified verbal autopsies (PCVA), external body examination, and collection of nasopharyngeal swab samples in cases of suspected COVID-19, with the 3 SVO-Mobile teams moving to their homes while continuing to receive bodies at headquarters.

From the 10th to the 31st epidemiological week (EW) of 2020, including March 1 to August 1, the number of household deaths in the city of Fortaleza attended by SVO-RF was 2,215, representing an increase of 69.00% to the same period of 2018 and 2019. The weekly percentage variation between the number of consultations in 2020 with the average of 2018 to 2019 varied from −14.56% in the 30th EW to +355.65% in the 19th EW (Table 1).

Among the 2,115 household deaths in this period, 353 (16.69%) cases had clinical–epidemiological criteria for suspected COVID-19, with the weekly variation starting from 0.00% in the first 2 weeks studied, up to 37.71% in the 20th EW (Table 1).

For any suspected case, nasopharyngeal swab samples were collected and sent to the Central Laboratory of Public Health of Ceará for the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) virus polymerase chain reaction technique in real-time (RT-PCR) testing. Among the suspected cases, the rate of positivity in the SARS-CoV-2 survey ranged from 0.00% at the beginning and end of the period analyzed to 100.00% positivity in the 18th EW (Table 1). It is a noteworthy fact that there were no confirmed cases of SARS-CoV-2 among home deaths in the last 5 weeks, which may represent both a decrease in the circulation of the

virus as well as the reestablishment of the capacity of the health services of providing medical assistance to possible new cases of the disease.

Therefore, in the light of an increase of up to 355.65% in the weekly number of home deaths, it was the ability to rapidly expand the investigation teams that made it possible to meet the excessive demand of these 2,115 deaths that occurred outside the hospital environment and confirm 280 deaths from COVID-19 that would hardly have been identified without this service (Table 1). Nevertheless, the use of the verbal autopsy did not allow the medical team to answer some questions about other home deaths that did not meet the criteria for suspicion for COVID-19. For example, would it be possible that all the excess of unsuspected deaths from COVID-19 resulted only from the difficulty of accessing health services? Or could some of these deaths have been caused by COVID-19 with an atypical clinical presentation without criteria for suspicion?

In view of these limitations, we believe it is necessary to invest also in other forms of investigation, such as minimally invasive necropsies, whose applicability and safety have already been demonstrated in other developing countries [7], with infectious diseases in general [8] and with COVID-19 specifically [9]. We also advocate the expansion of biosafety measures for the entire network of Death Verification Services, so that complete clinical necropsies can be performed even during pandemics, following the recommended good laboratory practices [10] and contributing to the understanding of the pathophysiological mechanisms that lead to death by COVID-19 [11] worldwide.

Despite these and other limitations of the verbal autopsy certified by a physician [12], we consider that, in a pandemic scenario, the investigation of home deaths by this method associated with the collection of samples for laboratory research constitutes a safe, financially viable, and secure method. It probably contributes to an increase in the detection of the disease and a consequent decrease in underreporting, especially in places where the collapse of the health system can lead to a rise in home deaths without medical assistance.

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Tropical Medicine and Infectious Disease

MDPI

Communication

High Effectiveness of SARS-CoV-2 Vaccines in Reducing COVID-19-Related Deaths in over 75-Year-Olds, Ceará State, Brazil

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Abstract: In Brazil, the SARS-CoV-2 vaccination program has so far prioritized people over 75 years of age. By the end of March 2021, in Ceará State, a total of 313,328 elderly people had received at least one dose of vaccine (45% Oxford-AstraZeneca/Fiocruz and 55% CoronaVac-Sinovac/Butantan), and 159,970 had received two doses (83% CoronaVac-Sinovac/Butantan and 17% Oxford-AstraZeneca/Fiocruz). After a single dose, there was already a significant reduction in COVID 19-related deaths (protection ratio: 19.31 (95% CI: 18.20–20.48), attributable protection ratio: 94.8%); higher protection ratios were observed after the application of two doses of the vaccine (132.67; 95% CI: 109.88–160.18), with an attributable protection ratio of 99.2%. SARS-CoV-2 vaccines are highly effective in reducing the number of COVID-19-related deaths in over 75-year-olds in Brazil, one of the hardest hit countries by the current pandemic.

Keywords: COVID-19; SARS-CoV-2; COVID-19 vaccines; mortality; epidemiology; public health

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1. Introduction

SARS-CoV-2 vaccines can reduce disease occurrence and transmission in a population. This is essential to reduce both morbidity and mortality from SARS-CoV-2 [1]. Consequently, there is a need for evidence on the effectiveness of vaccines to protect not only against SARS-CoV-2 symptoms but also to reduce COVID-19-related case fatality rates [2]. However, the reduction in the occurrence of severe disease and death is difficult to evaluate in phase 3 clinical trials, mainly due to the high number of participants required [1]. Thus, the effectiveness of SARS-CoV-2 vaccines in relation to case fatality has to be inferred from other sources of data, such as mortality statistics [3].

In Brazil, by the end of June 2021, more than 18.5 million cases and more than 500,000 deaths were confirmed, with a case fatality rate of 2.8% [4]. The state of Ceará, with a population of 8.8 million, was one of the first Brazilian states to confirm sustained transmission of COVID-19 in 2020 [5]. Despite the rapid implementation of control measures, Ceará stands out with more than 880,000 cases and almost 22,500 deaths by the end of June 2021 [4]. The case fatality rate was 2.5%, and there was a high rate of hospital bed occupancy (>90%), while different strains of SARS-CoV-2 were circulating [5].

A recent case-control study in England, including almost 160,000 adults aged over 70 years, evidenced a significant reduction in symptomatic COVID-19 cases and severe

symptoms after a single dose of the Oxford-AstraZeneca vaccine [6]. A recent study with Brazilian data showed an association between the rapid increase in vaccination coverage of the older population and relative mortality, as compared to younger individuals, in a setting where the gamma variant was predominant, and the most widely used vaccine was CoronaVac-Sinovac/Butantan [7]. The Brazilian Ministry of Health has made available both vaccines from Oxford-AstraZeneca/Fiocruz and CoronaVac-Sinovac/Butantan. In Ceará, by May 2021, more than 1.7 million people had taken at least one dose of a vaccine, with more than 500 thousand people having received two doses [8]. In this study, we evaluated the hypothesis that COVID-19 vaccinations had a considerable impact on reducing the number of deaths due to COVID-19 in the state of Ceará, Northeast Brazil, in the year 2021.

2. Materials and Methods

People aged 75 years or older were included since this age group was prioritized by the Brazilian Immunization Program and, thus, had a higher proportion of vaccination coverage at the beginning of the campaign. For the year 2021, the estimated population in this age group was 354,269 people (IBGE—Brazilian Institute of Geography and Statistics/*Instituto Brasileiro de Geografia e Estatística*).

We used data from the National Mortality System (SIM) and from the Immunization Program (SIPNI), between 17 January and 11 May 2021. The SIM database records all deaths that occur in Brazil. We selected death records with COVID-19 as the underlying cause of death. The SIPNI aims to coordinate immunization actions throughout Brazil, and records the immunobiological doses applied. The number of unvaccinated people was calculated as the difference between the estimated population and the number of vaccinated individuals. We included only individuals who had received at least one COVID-19 vaccine application. After removing duplicates, the databases were probabilistically related by means of people's names (*soundex*) and respective dates of birth, using Stata 15.1 software. The outcome was defined as people who died 21 days or later after the first dose of vaccine. We stratified the vaccinated population by number of doses, vaccine type and age group, and calculated the proportion of deaths as well as the protection ratio for deaths and percentage attributable protection ratio for deaths, and their respective 95% confidence intervals. All data in this study were extracted from secondary databases. The use of data was authorized by the Secretary of Health of the State of Ceará. As the study consisted of an analysis of secondary data, no informed consent was sought.

3. Results

A total of 313,328 elderly people (88.4% of the total population > 75 years) had received at least one dose of a vaccine, 44.5% from Oxford-AstraZeneca/Fiocruz and 55.5% from CoronaVac. A total of 159,970 had received two doses, 83.0% from CoronaVac-Sinovac/Butantan and 17.0% from Oxford-AstraZeneca/Fiocruz. The occurrence of deaths among the unvaccinated elderly was more than 132 times higher, as compared to those who had received two doses of a vaccine, with a protection ratio for deaths of 99.2%. After a single dose of a vaccine, the protection ratio was 19.3 (Table 1). The effect was more pronounced with increasing age.

Table 1. Protection ratios for death and percentage attributable protection ratios for deaths by COVID-19, stratified by number of doses applied, vaccine type and age group over 75 year-olds in the state of Ceará, Brazil, 2021.

Variables	N	Deaths	% Deaths	Protection Ratio (95% CI)	Attributable Protection Ratio (%) (95%CI)
Number of doses and type of vaccine:					
Oxford-AstraZeneca/Fiocruz 1st dose	139,322	716	0.51	17.91 (16.55–19.39)	94.4 (93.9–94.8)
CoronaVac-Sinovac/Butantan 1st dose	174,006	778	0.45	20.59 (19.07–22.22)	95.1 (94.7–95.5)
Vaccinated 1st dose	313,328	1494	0.48	19.31 (18.20–20.48)	94.8 (94.5–95.1)
Oxford-AstraZeneca/Fiocruz 1st and 2nd dose	27,193	3	0.01	834.45 (269.03–2588.18)	99.8 (99.6–99.9)
CoronaVac-Sinovac/Butantan 1st and 2nd dose	132,777	108	0.08	113.17 (93.50–136.99)	99.1 (98.9–99.3)
Vaccinated 1st and 2nd dose	159,970	111	0.07	132.67 (109.88–160.18	99.2 (99.1–99.4)
Not vaccinated	40,941	3769	9.21	1	-
Age Group–1st dose only:					
75 to 79 years					
Oxford-AstraZeneca/Fiocruz	32,749	141	0.43	8.39 (7.03–10.00)	88.0 (85.8–90.0)
CoronaVac-Sinovac/Butantan	97,072	481	0.50	7.29 (6.54–8.12)	86.3 (84.7–87.7)
Vaccinated	129,821	622	0.48	7.53 (6.82–8.33)	86.7 (85.3–88.0)
Not vaccinated	26,857	1010	3.76	1	-
80 to 89 years					
Oxford-AstraZeneca/Fiocruz	78,474	371	0.47	31.89 (28.59–35.58)	96.8 (96.5–97.2)
CoronaVac-Sinovac/Butantan	70,327	256	0.36	41.42 (36.42–47.12)	97.6 (97.2–97.9)
Vaccinated	148,801	627	0.42	35.78 (32.77–39.07)	97.2 (96.9–97.4)
Not vaccinated	13,336	2011	15.08	1	-
90 years or more					
Oxford-AstraZeneca/Fiocruz	28,099	204	0.73	137.74 (120.13–157.92)	99.2 (99.1–99.4)
CoronaVac-Sinovac/Butantan	6,607	41	0.62	161.14 (118.76–218.64)	99.3 (99.1–99.5)
Vaccinated	34,706	245	0.71	141.65 (125.04–160.48)	99.3 (99.2–99.4)
Not vaccinated	748	748	100.00	1	-

4. Discussion

Our data showed an impressive reduction in COVID-19-related deaths in older age groups in Ceará State, which is the population strata at highest risk for severe disease and death. Previous studies have shown that, by May 2021, more than 40,000 deaths had been prevented due to vaccination of the elderly population in Brazil with the Oxford-AstraZeneca and CoronaVac-Sinovac/Butantan vaccines [7]. Similar findings were found in the US after use of the first dose of Pfizer-BioNTech, particularly in older adults [9]. A study in Tennessee/USA showed a reduction of more than 95% in mortality in the vaccinated elderly population between December 2020 and March 2021 [10].

Considering the difficulties in the vaccine supply chain and their availability, it is important that the vaccines from both major producers showed a high effectiveness in reducing COVID-19-related deaths, even after a single dose. Furthermore, as predicted by Bolcato et al. in 2020, there may be problems that occur, such as insufficient production of vaccine doses for the entire population, or with different vaccination strategies and different times between doses, generating the need for difficult prioritization decisions [11]. In this context, the ability of a vaccine to protect against serious illness and death should be considered the most important outcome, since hospital admissions, especially in intensive care units, represent the greatest burden on health systems and has led several countries to face a collapse in their health systems. The global crisis generated by the coronavirus pandemic highlighted, once again, the importance of vaccination programs as effective public health measures, and brought about new mechanisms that may become models for future responses to regional epidemics and pandemics, with a greater variety of platforms and joint work to overcome challenges and accelerate vaccine development, manufacturing and delivery [12]. It is worth noting that the duration of protection after recovery from COVID-19 corresponds somewhat to the duration of protection provided by the vaccine [13].

For Hodgson et al. (2021), the beneficial effects of a vaccine can be assessed if the vaccine is effective in older adults and if there is a wide distribution of the vaccine [1]. The evaluation of asymptomatic SARS-CoV-2 infection is an important clinical outcome in the evaluation of vaccines, but is certainly of less public health importance than its effectiveness against death. In Italy, for example, the number of infections in nursing homes was particularly high, with a high mortality rate. Yet it must be recognized that the current situation of social disparity does not facilitate equal opportunities for all. As a result, the elderly will continue to experience moments of loneliness, despite efforts to reduce them [14].

Equal access to COVID-19 vaccines in all countries will continue to be a goal to be pursued. But the experience of previous pandemics suggests that access will be limited in low and middle income countries, despite the rapid development of some new candidate vaccines. Thus, the WHO proposal, with the COVAX Facility program, represents an attempt to facilitate multilateral cooperation to procure and distribute two billion doses of COVID-19 vaccines equitably in all countries of the world by the end of 2021 [15].

Our study is subject to some limitations, such as the use of secondary mortality data that may be subject to some errors. The smaller number of second doses by AstraZeneca in our study is basically due to the longer period between the two doses and, therefore, the population had not yet received the second dose during the study period. We also observed that the population of people vaccinated in the age group over 90 years was higher than the estimated population for this age group, this fact is due to the last census being conducted in 2010. We used the population projection for the year 2021, but there was still a difference of 1900 more people vaccinated in the population over 90 years of age. The estimated population was adjusted to the vaccinated population and, thus, data should be interpreted with care. Data on the antibody response of vaccinated individuals were not available, which may limit interpretation of results. However, we obtained population-based data from a population with a high vaccination coverage, and the study results can, thus, be considered as robust and valid.

5. Conclusions

SARS-CoV-2 vaccines are highly effective in reducing the number of COVID-19-related deaths in over 75 year-olds in Brazil, one of the hardest hit countries by the current pandemic.

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Informed Consent Statement: Patient consent was waived due to the research being conducted with secondary data from the immunization and mortality information systems. We obtained a declaration from the Ethical Review Board of the Federal University of Ceará (Fortaleza, Brazil) exempting us from the need for ethical clearance for this study. This exemption from ethical clearance is based on Brazilian laws: Law No. 12,527 of 18 November 2011 and National Health Council (CNS) Resolution No. 510 of 7 April 2016.

Data Availability Statement: The data presented in this study are publicly available in: https://integrasus.saude.ce.gov.br/#/indicadores/indicadores-coronavirus/indice-transparencia (accessed on 13 July 2021).

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Comparative Morphological Characterization of Skin after Subcutaneous Application of Hyaluronic Acid and Polycaprolactone in Rats: Establishment of an Experimental Model

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Abstract

Injectable facial fillers are excellent options for treating facial aging, wrinkles, and contour defects. Both polycaprolactone (PCL) and hyaluronic acid (HA) have been used to restore lost tissue volume and improve facial contour. However, the mechanisms involved in the effect of these biomaterials still need to be fully understood. The present work aims to establish an experimental model to investigate cellular and morphological changes in the skin of Wistar rats in response to HA and PCL to understand the mechanisms associated with these effects. The subcutaneous tissue of the back of Wistar rats was used as a reception area for biomaterials, represented by the commercial products Ellansé®, containing polycaprolactone (PCL) and Juvederm Voluma®, containing hyaluronic acid (HA). Animals were euthanized afetr 30 or 60 days, and skin samples were collected from treated and untreated animals (CONTROL) for histological and immunohistochemical evaluation for IBA-1, TGF-β, and FGF. Analysis of type I and type III collagen deposition, neovascularization, and adipose tissue was performed. On histological examination, HA appeared as an amorphous, basophilic material interspersed with connective tissue bundles. The skin fragments with PCL showed intense cell proliferation, with foreign body giant cells and a higher capillary proliferation than the HA group. More vessels were observed in the HA and PCL groups compared to the CONTROL group. A significant increase in fibroblasts and fibrocytes was observed in skin fragments inoculated with HA and PCL, associated with increased FGF expression. The number of fibroblasts was significantly higher in the PCL group than HA. The PCL group showed higher immunostaining for IBA-1 and TGF-β than the CONTROL and HA groups. Collagen deposition was observed in the treated groups, especially type III collagen in the PCL group, when compared to HA. Our morphological results demonstrated stimulation of fibroblastic activity and active-related tissue regeneration, with increased vascular proliferation and expression of markers related to tissue proliferation, mainly associated with the PCL group. We also observed increased adipose tissue, although further studies are needed to confirm these findings.

1 INTRODUCTION

Like other organs, the skin undergoes a natural aging process over time. Moreover, it is directly exposed to environmental damage, especially ultraviolet radiation (UV)[1]. Because of this process, contour deficiencies, wrinkles, loss of dermal matrix, and decreases in collagen and elastic fibers appear, resulting in a reduction of elasticity [2–4]. In addition to skin aging, three-dimensional losses are seen in the face, involving bones, muscles, and fat pads that affect the aged appearance. Many dermal fillers have been used to minimize these losses, filling in wrinkles and restoring the volume of tissue lost, either due to disease or aging [5]. Soft tissue fillers are less invasive with less downtime than traditional surgical interventions[6]. One of the most used dermal fillers is hyaluronic acid (HA) due to its biocompatibility, non-immunogenicity, biodegradability, and high-water absorption capacity. HA performs different functions, such as lubrication, hydration, and maintenance of tissue structure. In addition, it is involved in cell proliferation and migration events, as well as angiogenesis [7].

Another material used for filling purposes is polycaprolactone (PCL), a polymer that provides safe and lasting correction of aging-related volume loss or changes in facial contour. The main aesthetic results obtained with PCL are volume restoration, contour redefinitions, and wrinkle reduction. Its positive effect on skin quality has also been widely reported. The safety profile, easy injection, and adjustable longevity are determining factors in choosing this product [7, 8]

Substances that restore lost tissue volumes, such as HA and PCL, are highlighted in this context. However, the mechanisms involved in the effect of these materials still need to be fully understood. Therefore, the present work aims to establish an experimental model to investigate the morphological changes in the skin after PCL and HA injections through histomorphometry and autofluorescence techniques.

2 METHODS

2.1 Study design and ethical aspects

The present study was randomized, controlled, and blinded. The Guidelines conducted surgical procedures and animal treatments for Institutional and Animal Care and Use (CEUA) of the Federal University of Ceará, Brazil (#7904140420 / 2020).

2.2 Animals

Sixteen heterogeneous male Wistar rats, 200-250g, from the UFC central vivarium, were maintained in an environment-controlled room with a 12-h light/12-h dark cycle. The animals were kept in standard plastic cages with metal lids, with four animals per cage, and received water and food *ad libitum*. Every effort was made to reduce the number of animals used and their pain, suffering, and stress.

Preparation of animals

2.3 Dermal Fillers

In the present study, Juvéderm Voluma® (Allergan®) and Ellansé® (Sinclair Pharma, London, UK) fillers were used. Juvéderm Voluma® is a homogenized dermal filler composed of cross-linked hyaluronic acid at 20 mg/ml concentration in a physiological buffer with lidocaine. Juvéderm Voluma® is derived from Streptococcus equi and cross-linked with diglycidyl ether 1,4-butanediol (BDDE) in a one-step cross-linking process in which high and low molecular weight molecules are mixed. Ellansé® is a PCL-based collagen stimulator with 30% synthetic PCL microspheres suspended in 70% aqueous carboxymethyl cellulose.

2.4. Experimental model for the injection of dermal fillers

The animals were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), and, in the following, the animals' backs were trichotomized. Samples were injected in boluses of 0.05 ml, containing PCL or HA, in the subcutaneous tissue of the animals' backs, just below the panniculus

carnosus, using a 26G needle. Each animal received both biomaterials injected in two different points of the dorsal-lateral region, respecting a minimum distance of 5cm between them. They were followed up daily for seven days to verify signs of acute inflammation at the inoculation sites. The animals were euthanized with an overdose of 2% xylazine (30mg/kg) and 10% ketamine (240mg/kg) thirty (n = 8) or sixty (n = 8) days after the injection of the fillers. The skin on the back of each animal was folded back, allowing visual inspection of the subcutaneous tissue with the fillers. Signs of inflammation and fibrosis were investigated, and the volume gain related to each filler was observed. Next, skin samples containing HA, PCL, as well as skin samples with no fillers (CONTROL) were surgically removed and divided into two fragments: one fixed in 10% buffered formalin and the other stored at -80°C for further processing for histological slides and protein extraction, respectively.

2.5 Histopathological analysis

The tissues fixed in 10% buffered formalin were processed for paraffin embedding. Histological slides were prepared using 4µm thick tissue sections, stained with hematoxylin and eosin (HE), Mallory's trichrome, and picrosirius red. An experienced pathologist, who did not know which were the control or treatment samples, performed all histological analyses. HE stained sections were qualitatively evaluated in at least 6 (six) specimens *per* group, considering five fields per slide, at a magnification of 200x. Inflammatory findings such as cellular infiltrate, edema, and hemorrhage were investigated. We also counted fibroblasts and fibrocytes in at least six slides of each group, HA, PCL, and CONTROL, considering five fields per slide at 400x magnification. As for the slides stained with Mallory's Trichrome, the effect of biomaterials on the connective tissue vasculature at the periphery of the implants was investigated by quantifying the number of blood vessels and the total vessel area. At least six specimens per group were used. Initially, the areas of greatest vascularization were identified (hot spots) for delimitation and measurement of the areas of the ten largest blood vessels, considering the cross-sectional diameter, from digitalized images. The images were captured using a digital camera attached to a Leica microscope at 400x magnification and analyzed with LAS V3.7.0 software [9]

The quantification of the vessels was also performed by observing five fields per slide and six specimens per group, at 200X magnification. The slides stained with picrosirius red were photographed with a DFC 295 camera attached to the optical microscope under polarized light (Leica DM 2000). Evaluation of type I and III collagen fiber deposition was performed at the periphery of the biomaterials after light polarization, allowing differentiation of type I (yellowish orange to orange and red birefringence) and type III (green or yellow-green birefringence) collagen. From each slide, 10 histological fields were selected and photomicrographed at 400x magnification. The images were analyzed using the computational program Photoshop to obtain the collagen percentage by automated particle analysis according to the selection and measurement of the areas based on color. The values for each collagen type in Threshold Color were standardized for all images.

2.6 Immunohistochemical analysis

Immunohistochemical analyses for IBA-1, TGF-β and FGF were performed in CONTROL, PCL, and HA groups by the streptavidin-biotin peroxidase method in formalin [10]. Tissue sections were fixed in paraffin-embedded, cut four μm thick, and prepared in poly-L-lysine-coated microscope slides. After deparaffinization, antigenic recovery was performed with citrate buffer recovery solution (pH 6.0) for 20 minutes at 95°C. The endogenous peroxidase was blocked with 3% H2O2 for 10 minutes to reduce non-specific binding. The sections were then incubated with anti-IBA-1 (dilution 1: 100; Santacruz Biotechnology; California, USA), anti-TGFβ (dilution 1: 100; Santacruz Biotechnology; California, USA), anti-FGF (dilution 1: 100; Santacruz Biotechnology; California, USA) diluted in DAKO antibody diluent for 1 h. The antibody binding sites were visualized by incubation with diaminobenzidine-H2O2 solution (DAB, DAKO; California, USA). In parallel, negative control without the primary antibody was performed. The slides were counterstained with hematoxylin, dehydrated in graded alcohol series, clarified in xylene, and covered with a coverslip. Positivity for IBA-1, TGF-β, and FGF was determined by brown staining at the cytoplasmic level in the connective tissue. Cytoplasmic quantification of immunolabeled cells was performed on at least 6 slides per group. From each slide, 5 histological fields (400x magnification) were selected and photomicrographed with a digital camera attached to an optical microscope (Leica DM 2000, Wetzlar, Germany). The images were analyzed using the computational program Photoshop 8.0 to quantify the percentage of immunolabeled area. The percentage of the immunolabeled area was calculated from the ratio between the immunolabeled area (in pixels) multiplied by 100 and the total area (in pixels), as previously described [11]

2.7 Western blot analyses

First, 20μg of protein of each sample was prepared from frozen tissue by adding sample buffer (BioRad, USA 65.8 mM Tris-HCl, pH 6.8; 26.3% glycerol; 2.1% SDS; 0.01% bromophenol blue) and βmercaptoethanoll (BioRad, USA) and vortexing for 10 seconds. Plus, heating in a water bath (95°C, 5 min) and spinning (10000 rpm, 4°C, 30s). Next, vertical polyacrylamide-SDS gel electrophoresis of proteins (SDS-PAGE) was performed at 60 v for the first 15 min for deposition of samples at the bottom of the well; and 120 v for the rest of the run, where 10% gel (FGF, and β-actin) and race buffer (25 mM Tris; 192 mM glycine; 1% SDS) were used. After the run, transfer of the proteins from the gel to the PVDF membrane (BioRad, USA, Polyvinylidene Fluoride) was performed at 100-fold for two hours in transfer buffer (25 mM Tris; 192 mM glycine; 20% methanol). After this step, the membranes were blocked for one hour under constant agitation, to reduce nonspecific binding, with 5% BSA (Sigma-Aldrich, USA) diluted in Tris-HCl saline buffer supplemented with Tween 20 (TBST- 20 mM Tris pH 7.5; 150 mM NaCl; 0.1% Tween 20). Next, the membranes were washed with TBST, with three washes for 10 min each. In the next step, the membranes were incubated overnight at 4°C under constant agitation, with the FGF (1:100; Invitrogen, USA) or anti-β-actin (1:200; Santa Cruz, USA) antibodies diluted in 1% BSA in TBST. After this step, three washes of 10 min each with TBST were performed. The membranes were incubated with the HRP-goat anti-rabbit secondary antibodies (1:1000; Invitrogen, USA) for two hours at room temperature. After this time, the membranes were washed thrice with 10 min duration each, using TBST. Chemiluminescence reagent (BioRad, USA, Clarity western ECL blotting substrate) was added, and the membranes were shaken for 5 min. Images of the bands were captured by a ChemiDoc XRS system (BioRad, USA) or

exposed to radiographic film. The density of the bands was measured using ImageJ software (NIH, Bethesda, MD, USA).

2.8 Statistical analysis

Data are presented as mean ± SEM. Analysis of variance (ANOVA) was followed by Tukey's test, using GraphPrism (GraphPad 6.0 Software Inc., La Jolla, CA, USA). The significance level adopted was ($p < 0.05\%$) for all analyses.

3 RESULTS

3.1 Visual inspection of changes induced by HA and PCL in the skin of *Wistar* rats.

When the animal's posterior skin was folded (Fig. 1A), it was possible to macroscopically observe an increase in tissue volume at the points where the fillers were applied, suggesting the presence of PCL (Fig. 1B) and HA (Fig. 1C) in the subcutaneous tissue. The HA recipient point was nodular and translucent, with an elastic consistency and a smooth surface. The surface was homogeneous and elastic when the tissue was cut into two fragments. The spot that received PCL inoculation had irregular, slightly raised, whitish edges with a firm consistency and a granular surface when cut.

3.2 Microscopic changes induced by HA and PCL in the skin of *Wistar* rats.

The biological response to the administration of the biomaterials was examined histologically to investigate the effects of HA and PCL. Figure 1D shows the stratification of the rodent skin with the application of the biomaterials below the panniculus carnosus. Thirty days after the subcutaneous administration, the HA group presented as an amorphous, slightly basophilic material, dissociating fat lobes (Fig. 1F, 1I). The biomaterial (HA) was also intercalated with connective tissue bundles. No histologically apparent tissue reaction was observed, except for small, sporadic nuclear aggregates compatible with foreign giant body cells and the presence of adipose tissue at the periphery of the material, as illustrated in Fig. 1O. In some areas, a discrete capillary proliferation was also observed, always associated with the presence of the injected substance.

In the PCL samples, the marked cellular proliferation of cells with granular cytoplasm, nuclei mostly eccentric, small, round, and with fine chromatin compatible with macrophages, was observed. The increase of small vessels was observed, as well as loose connective tissue, with focal areas showing thicker collagen bundles, demonstrating moderate fibrosis (Figs. 1G, 1J). This connective tissue was found between the material particles at the periphery of the PCL mass. After sixty days, small capillaries persisted in HA, but with a more regular distribution, without denoting considerable tissue reaction (Fig. 1L). In the samples with PCL, there was less cellularity, fewer granular cells, more fibrous connective

tissue between the material particles, and more closely spaced cells with bundles of collagen fibers (Fig. 1M). The presence of capillaries was maintained when compared to 30 days.

3.3 Effect of biomaterials on cell proliferation and neovascularization

The presence of nuclear aggregates and fusion of epithelioid cells, which is compatible with activated macrophages, were observed by immunoglobulin analysis for IBA-1 (Fig. 2A), characterized by marking cells with brownish staining in the skin of mice subjected to HA and PCL injection. After 30 days of biomaterial administration, this analysis showed a significant increase in activated macrophages in HA and PCL compared to the CONTROL group and a higher presence of activated macrophages in PCL compared to HA ($p < 0.05$). After 60 days, the pattern of macrophage activity showed a slight reduction in the treated groups, in which only the HA group had a significant decrease in its activity compared to the 30 and 60-day periods.

Immunohistochemical analysis showed, after 30 days, a significant increase in TGF-β immunohistochemistry in the HA and PCL groups compared to the CONTROL group. Still, there was no statistical difference between them. After 60 days, there was a significant decrease in immunolabeling in the HA and PCL groups compared to the previous period (30 days). A statistical difference was observed between the two treated groups, in which the PCL group had higher labeling than the HA group in the same period, as illustrated in the graph in Fig. 2B.

The vascular proliferation observed on the HE-stained slides was quantified using Mallory's Trichrome staining (Fig. 2C), which allowed the measurement of vessel area and number. As for the number of vessels, after 30 days of the injection period, a statistically significant increase was observed in both the HE and PCL groups compared to the control group. In addition, the PCL group showed an even more substantial number of vessels than HA ($p < 0.05$). After 60 days, there was a reduction in the number of vessels in the experimental groups, HA and PCL. However, there was still a significant increase in vessels in these groups when compared to the CONTROL group, which can be seen in the graph in Fig. 2C. However, no effect of biomaterials on vessel area was observed in any of the periods studied, as seen in the graph and illustrated in the images (Fig. 2C).

The histopathological analysis also showed an increase in the number of fibroblasts and fibrocytes, especially in the first 30 days after the injection of the biomaterials. The increase was significant for both cell types in the HA and PCL groups compared to CONTROL ($p < 0.05$). A more significant number of these cells was observed in the PCL group compared to -HA ($p < 0.05$) in both periods, followed in Fig. 3B.

A. Immunostaining for FGF at 30 and 60 days and quantification of 1 expressed in the graph. **B.** Fibrocyte and fibroblast count at 30 and 60 days represented in the graph. **C.** Illustrative image of the fibrocyte and fibroblast in the connective tissue adjacent to the biomaterial. Bars show mean ± SEM of 6 animals per group. * represents the significant difference of HA and PCL when compared to CONTROL, at 30 and 60

days. # represents the significant difference between PCL and HA. (*p < 0.001), ANOVA followed by Tukey's test).

The immunohistochemical analysis for FGF showed that after 30 days, there was a significant increase in the immunoexpression of this cytokine in the PCL group when compared to CONTROL and HA, as can be seen in Fig. 3C and confirmed in the representative images of brownish staining in the cytoplasm of the fibroblasts. As for the 60 days, the immunostaining for FGF became higher in both treated groups compared to CONTROL. However, no statistical difference was observed between them, which can be seen in the graph of Fig. 3A, which shows the quantification of these cells and the protein expression of FGF.

Collagen deposition in the biomaterial-treated skin was evaluated after 30 and 60 days using histological sections stained with picrosirius red, observed under a polarized light microscope. No significant difference was observed in the deposition of type I collagen in the periods evaluated, nor between the groups. Organized reddish-yellow fibers represent this collagen in both HA and PCL groups ($p < 0.001$) (Fig. 4) In these groups, a dominance of type III collagen, represented by green fibers, was observed, as shown in the illustrations and confirmed in the graphs quantifying the areas marked in red and green, representing type I and III collagen, respectively.

4. DISCUSSION

Fillers are the most widely used substances in aesthetic medicine [12] and are a viable alternative to surgery for patients seeking a safe, minimally invasive, and affordable means of maintaining a youthful appearance and correcting facial contour deficiencies[13]. These products have become a staple in aesthetic and medical procedures. A wide variety of options are available on the market, but the mechanisms associated with their effects still need to be fully understood [14]

It is known that the physiological reactions induced by these biomaterials are conditioned by their physical and biochemical properties, patient characteristics, and the injection technique used [15]. To better understand the biological interaction played by fillers, rodent models have proven to be a helpful tool, assessing, in addition to morphological changes in the skin, the lifting capacity, and the resistance to tissue deformation [16].

Traditionally, these experimental models inoculate the biomaterials into the dermal layer [17–20], one of the injection planes used to apply fillers. However, for safety purposes, most practitioners in the clinic use blunt cannulas for the injections, making filling at the dermal level impractical. The injection plane becomes subcutaneous with a blunt cannula, a trend in use today [21]. For this reason, the experimental model standardized in the present study evaluates the effects of the subcutaneous application of fillers.

Rodent skin differs from human skin, which must be considered in experimental studies. In rodents, the epidermis is thin and has a high density of hair follicles. The dermal white adipose tissue is delicate and lies directly beneath the reticular dermis, clearly separated from the subcutaneous white adipose tissue

by a muscular layer called the panniculus carnosus [22]. Although many other mammals, including humans, do not have the panniculus carnosus, layers of adipose tissue exist beneath the reticular dermis in several species, including pigs and humans [23]

Considering the proposal of applying fillers at the subcutaneous level, we initiated the development and standardization of our experimental model, evaluating morphological changes in Wistar rats' skin after using hyaluronic acid (HA) and polycaprolactone (PCL). The increasing worldwide demand for soft tissue fillers motivated us to investigate the direct action of these materials on intimal tissue.

Visual inspection of the rat's skin after euthanasia and histopathological analysis in both periods evaluated confirmed the biocompatibility with no clinical, macro, or microscopic signs of inflammation. The folded skin showed that the HA had a nodular appearance, and its volume appeared to increase after 30 days of biomaterial inoculation. In tissues with PCL, there was volume loss after 30 and 60 days, visually observed right after the Ellansé application. With 70% CMC in its composition, Ellansé, presents a general decrease in volume due to the reabsorption of this vehicle after one month. Connective tissue and neoformed vessels replace approximately 50% of the original CMC volume. The key to maintaining volume after injection of PCL-based filler is the formation of new vessels and collagen deposition [24], as observed in the present study, suggesting the volumizing capacity of PCL is smaller than hyaluronic acid's capacity.

According to the literature, HA promotes volumization and has an essential hygroscopic function, contributing to volume gain[25]. Histological analysis confirmed the presence of the biomaterials in the subcutaneous tissue below the panniculus carnosus skeletal muscle layer. The HA group showed mild neovascularization and an apparent increase in adipose tissue in the peri-implant region. Some authors consider HA to be minimally immunogenic, making it the most commonly used temporary filler with clinically satisfactory results in terms of volume. In the present study, this fact is corroborated by the increase in the volume of the area that received HA, 30 and 60 days after application. Hyaluronic acid dermal fillers are now considered the preferred material for minimally invasive cosmetic interventions [26]. Because HA does not show specificity for any organ or species, HA is considered immunologically inert [27, 28]. According to papers published in the literature, once injected into the skin, HA causes a mild inflammatory reaction at the host tissue boundary followed by a gradual fibrous growth, which anchors the gel to the surrounding host tissue, preventing product migration [29]. Our macroscopic and histological analyses after 30 and 60 days did not detect any evidence of acute inflammation. We observed that HA induces a discrete formation of fibrous connective tissue, which probably contributes to preventing material displacement. In addition, we observed an increase in adipose tissue at the periphery of the HA; however, no measurements were taken, and further studies are needed to confirm this finding. In 2020, Nadra and colleagues demonstrated in *in vitro* studies that treatment with cross-linked HA showed beneficial effects on cell adhesion and survival and reduced basal and induced lipolysis in fully mature adipocytes.

In this work, cross-linked HA promoted cell adhesion and preserved the adipogenic capacity of pre-adipocytes during prolonged cell culture, bringing additional evidence of the beneficial role of cross-linked HA-based fillers in maintaining subcutaneous fat. On the other hand, another paper published in 2017 demonstrated that HA promoted the proliferation of adipose tissue-derived stem cells and the differentiation of these cells into adipocytes, suggesting an action of HA related to increasing the number of adipocytes [30]. The presence of adipose tissue associated with PCL, appeared less evident when compared to HA and more apparent when compared to CONTROL. However, further studies are needed to confirm this finding. We found no evidence in the literature to support this finding. When analyzing the PCL samples, we identified, among the particles of the material, intense cell proliferation, and neovascularization, besides deposition of extracellular matrix between the particles and discrete collagen deposition. Some foci of the CMC carrier were also observed at 30 and 60 days. After 60 days, the cellularity decreased, showing an apparent increase in collagen deposition between the particles when compared to the 30-day analysis. There are reports in the literature that the CMC itself, the carrier of the PCL particles, seems to stimulate the tissue reaction until the complete resorption of the biomaterial is found in some giant cells in its periphery [31]. It is well established in the literature that the presence of biomaterial can induce a foreign body reaction, where monocytes migrate into the tissue, becoming macrophages, which, together with platelets, synthesize platelet-derived growth factor (PDGF) and transforming growth factor beta (TGFβ), which promote the migration of fibroblasts [32]. It was observed, through immunohistochemistry for IBA-1, the appearance of a higher number of activated macrophages in the experimental HA and PCL groups when compared to the control group (CONTROL). This increase was more expressive in the 30-day evaluation, with a more significant number of activated macrophages in the samples with PCL compared to HA, and both (HA and PCL) showed greater immunolabeling for IBA-1 when compared to the CONTROL group. There was a significant reduction of activated macrophages in the HA group at 60 days, but the statistical difference concerning the CONTROL group was maintained. The number of activated macrophages in the PCL group remained high after 60 days, and there was no significant difference in IBA-1 immunolabeling between the 30 and 60-day analyses. The higher number of macrophages in this group is probably related to the physical characteristics of the polycaprolactone, which presents as immunologically inert microspheres large enough to induce macrophage aggregation.

As mentioned earlier, macrophages release TGF-β, regulating cell behavior. TGF-β is known to be a potent chemoattractant for endothelial cells and fibroblasts, as well as for cells of innate immunity, such as neutrophils and monocytes[33], TGF-β can be considered an essential physiological regulator both for the maintenance of the extracellular matrix and also in tissue repair processes [34]. In the present study, we observed significant differences in immunostaining for TGF-β between the HA and PCL groups compared to the CONTROL group in the 30 and 60-day analyses. A substantial increase in TGF-β expression was observed in the 60-day analyses in the PCL group compared to HA, which was not observed at 30 days. Immunolabeling was observed mainly in the cytoplasm of macrophages. The significant increase in TGF-β immunolabeling in the PCL group compared to HA after 60 days is related to the higher expression of IBA-1 observed in PCL since the expression of TGF-β by macrophages is well established in the literature.

Moreover, the significant increase of fibroblasts in HA and PCL groups, compared to CONTROL, also contributes to understanding the higher expression of TGF-β in these groups. Although macrophages are the primary source of TGF-β, studies demonstrate fibroblasts' expression of this growth factor, especially during repair processes [35], closing a cycle in which TGF-β expressed by macrophages is a potent chemoattractant for fibroblasts, which in turn can express TGF-β.

The present study observed a significant increase in fibroblasts and fibrocytes in the HA and PCL groups compared to CONTROL at the evaluated periods. Fibroblasts are the most abundant cells in the dermis. These cells' essential characteristics are their ability to synthesize and remodel ECM. In a repair process, fillers work as a framework for the proliferation of these cells, which are the primary basis for fibrogenesis [36]. The literature further describes the chemoattractant role of TGF-β for endothelial cells [37]. Our study observed a significant increase in small vessels and capillaries in the HA and PCL groups, 30 days after inoculations compared to CONTROL. In contrast to that observed in HA, The number of vessels in the PCL group remained significantly higher compared to CONTROL even after 60 days. This result can be justified, at least in part, by the maintenance of high TGF-β expression observed in the PCL group even after 60 days. Despite the more significant number of vessels observed in the HA and PCL groups, there were no statistical differences regarding vessel area between the experimental groups, probably due to the small cross-section of the capillaries. We observed increased protein expression, by immunohistochemistry and Western Blot, for fibroblast growth factor (FGF) in both HA and PCL groups compared to the CONTROL group. Fibroblast growth factors (FGFs) are broad-spectrum mitogens and regulate cellular functions, including migration, proliferation, differentiation, and survival. FGF signaling is essential in tissue development, metabolism, and homeostasis [38]. FGF family members increase fibroblast proliferation and activation, stimulating collagen accumulation and angiogenesis, and are essential in tissue repair [39–41].

Our data reinforce these studies since the higher protein expression of FGF was accompanied by a significant increase in fibroblasts, blood vessels, and collagen in tissue inoculated by both fillers evaluated. Collagen deposition was assessed in the connective tissue adjacent to the HA and PCL mass by Picrosirius Red staining under polarized light. There was a more significant deposition of collagen III, at 30 days, in PCL compared to HA, probably related to the increase of FGF already in this period. In 60 days, there was a significant reduction of collagen III in groups HA and PCL compared to the previous period, with no significant difference between HA and PCL. When type I collagen was evaluated, there were no differences between HA and PCL in the periods considered. It is known that collagen is the dominant component of the ECM in the dermis and accounts for approximately 70% of its dry weight.

Furthermore, in intact adult skin, the ratio of collagen I to collagen III is approximately 4: 1. The amount of collagen III increases temporarily when the skin is injured and during neoderm formation. In freshly healed human skin, the ratio of collagen I to collagen III is about 1: 1, as in neonatal skin. At the same time, in response to a wound, the skin may have a higher amount of collagen III and hyaluronic acid and a lower amount of collagen I [42]. The higher amount of collagen III observed in our analyses is probably associated with the natural tissue repair and healing process, which initially forms collagen III.

In 2014, Kim and colleagues investigated whether PCL-based dermal filler induced neocollagenesis in human tissue in a pilot study by histological analysis. Two patients indicated for temple lift surgery were included in the study. PCL was injected intradermally into the temporal region, just below the hairline, which would be included in lift surgery, 13 months after injection. Tissue collected after surgery showed collagen formation around the PCL particles, maintained even 13 months after injection [31].

Another study compared neocollagenesis and elastin production stimulated by Radiesse® (calcium hydroxylapatite; CHAA, Merz Pharmaceuticals GmbH) and Juvéderm® VOLUMA®, the same HA used in the present study. Twenty-four women received subperiosteal injections in the retroauricular region, and punch biopsies were performed 4 and 9 months after the injections. The authors noted that type I collagen gradually replaced type III collagen after 9 months of injection [43]. An animal study using Ellansé showed the formation of type III and type I collagen after nine months of biomaterial injection. After 21 months, the predominance of type I collagen deposited around the PCL microspheres suggests that type I collagen replaces type III collagen in the long term, such as in wound healing [20]. Based on these studies, we can deduce that with a more extended observation of the inoculated animals, we would detect a more significant amount of collagen I in our samples.

Our data reinforce literature studies showing the benefits of temporary fillers in maintaining skin volume. Although HA had a more significant volumizing effect, PCL stimulated greater collagen deposition after 30 days compared to HA. This result is reinforced by the higher number of fibroblasts observed in the PCL group. Therefore, we speculate that the greater volumization observed in the HA group is mainly due to its hygroscopic action, an already well-established effect in the literature. Ideally, in applying biocompatible materials, no exacerbated tissue reaction should be adjacent to the injected product. Mild, controlled, subclinical inflammation is expected to prolong the product's longevity. The local response of the tissue to the foreign body, through phagocytosis, is the most critical factor in determining the filler's longevity. In this process, some enzymes are present in the tissue, and free radicals break the filler into fragments that are removed by circulating macrophages and, subsequently, by lymphatic channels [44].

5. CONCLUSION

The standardized model for evaluating subcutaneous fillers proved viable, mimicking the injection plan used in humans and allowing the analysis of the cellular and morphological alterations proposed in this study. Our morphological findings demonstrate the stimulation of fibroblast activity and a related active regeneration of the connective tissue, with increased vascular proliferation and expression of markers FGF and TGF-β, related to tissue proliferation, especially in the PCL group. We observed increased adipose tissue related to the treated groups. However, no quantification was done to measure this increase, and further studies are needed to confirm this finding and investigate the role of the adipose tissue when in contact with HA and PCL. The study indicates the period evaluated induced collagen deposition, mainly type III.

Declarations

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Informed consent: Not applicable to research involving animals.

Conflict of Interest: The authors declare no competing interests.

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Figures

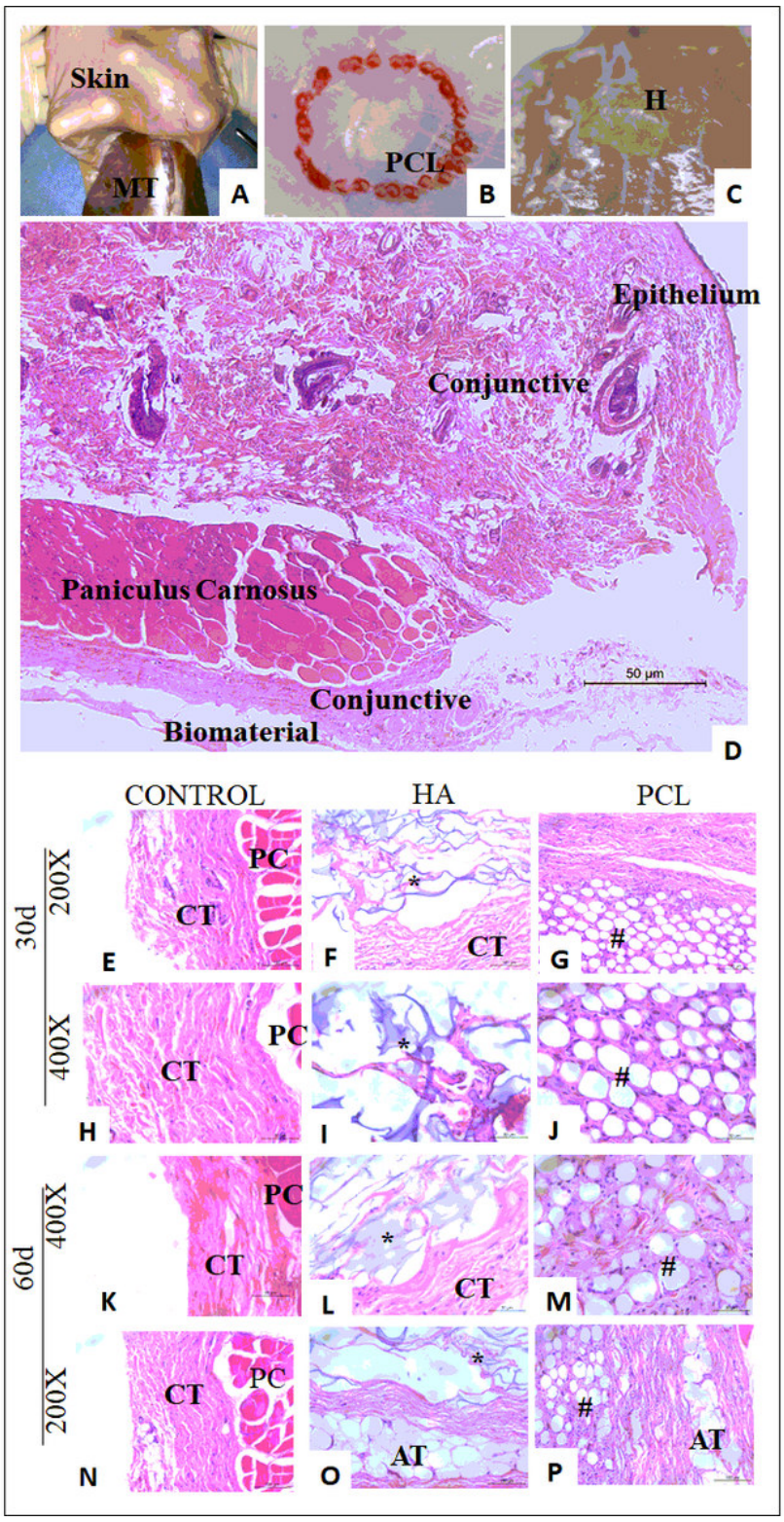

Figure 1

Macroscopy and microscopy of rat skin after injection of PCL (#) and HA (*). **A**, The animal's skin reveals muscle tissue (MT) **B**, Visual inspection of PCL. **C**, Visual inspection of HA. **D**, Microscopic topography of the mouse skin highlights panniculus carnosus (40x magnification). **E**, **H**, **K**are photomicrographs of untreated skin at 30 and 60 days (CONTROL group). **F**,**I**, and **L** are photomicrographs of HA-treated skin.

G, **J**, and **M** are photomicrographs of PCL-treated skin. **N**, **O**, and **P**show the presence of adipose tissue (AT) in the loose connective tissue (CT), below the panniculus carnosus, in the HA and PCL groups.

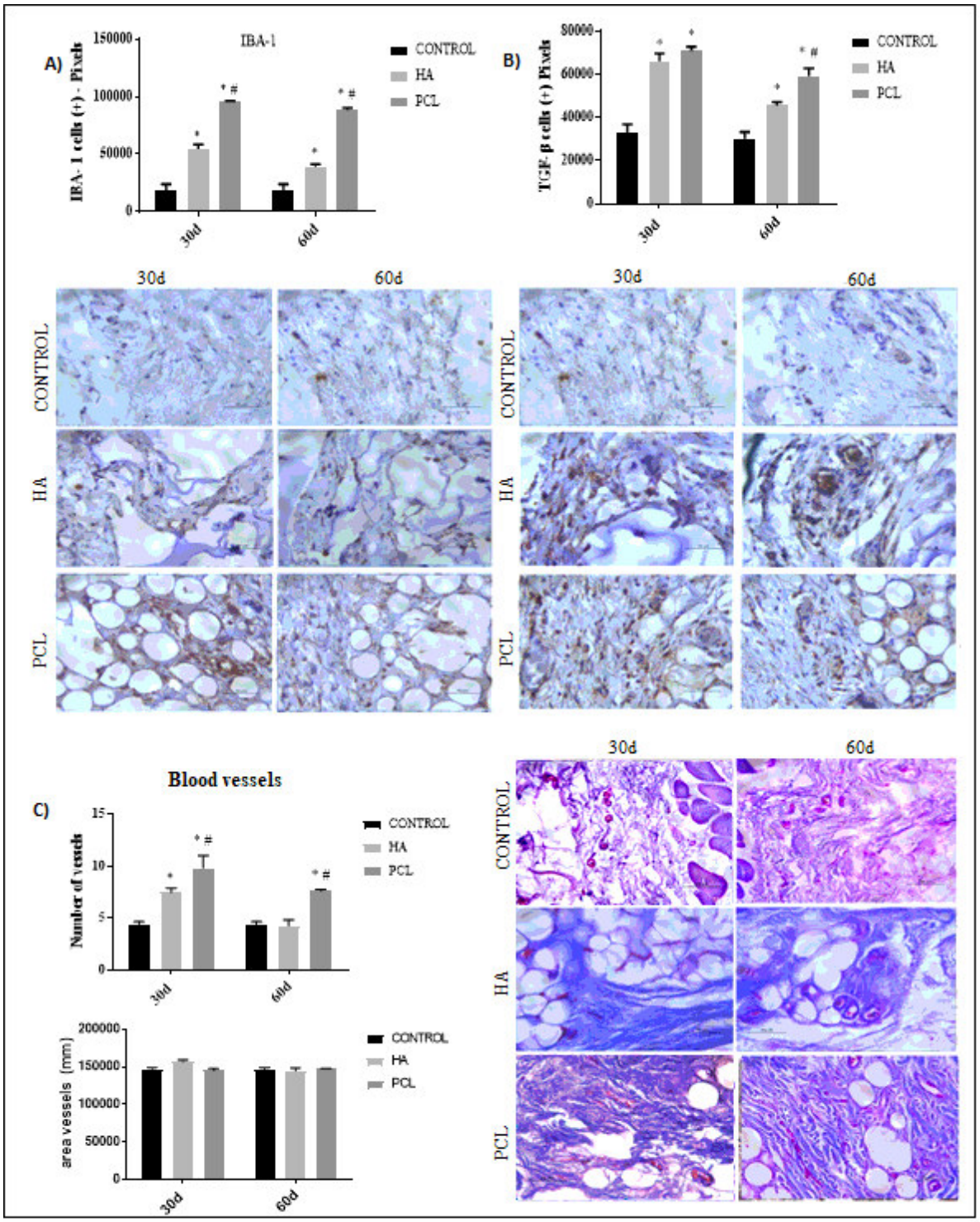

Figure 2

Immunohistochemistry for IBA-1, TGF-β, and analysis of neovascularization using Mallory's Trichrome. **A**. Immunostaining for IBA-1 at 30 and 60 days and quantifying activated macrophages. **B**. immunostaining

for TGF-β at 30 and 60 days and quantification expressed in the graph. **C**. Measurement of the area of the vessels on the periphery of the biomaterials and quantification of vessels after 30 and 60 days. Bars show mean ± SEM of 6 animals per group. * Represents the significant difference in HA and PCL compared to CONTROL at 30 and 60 days. # Represents the significant difference between PCL and HA. (*p< 0.001), ANOVA followed by Tukey's test).

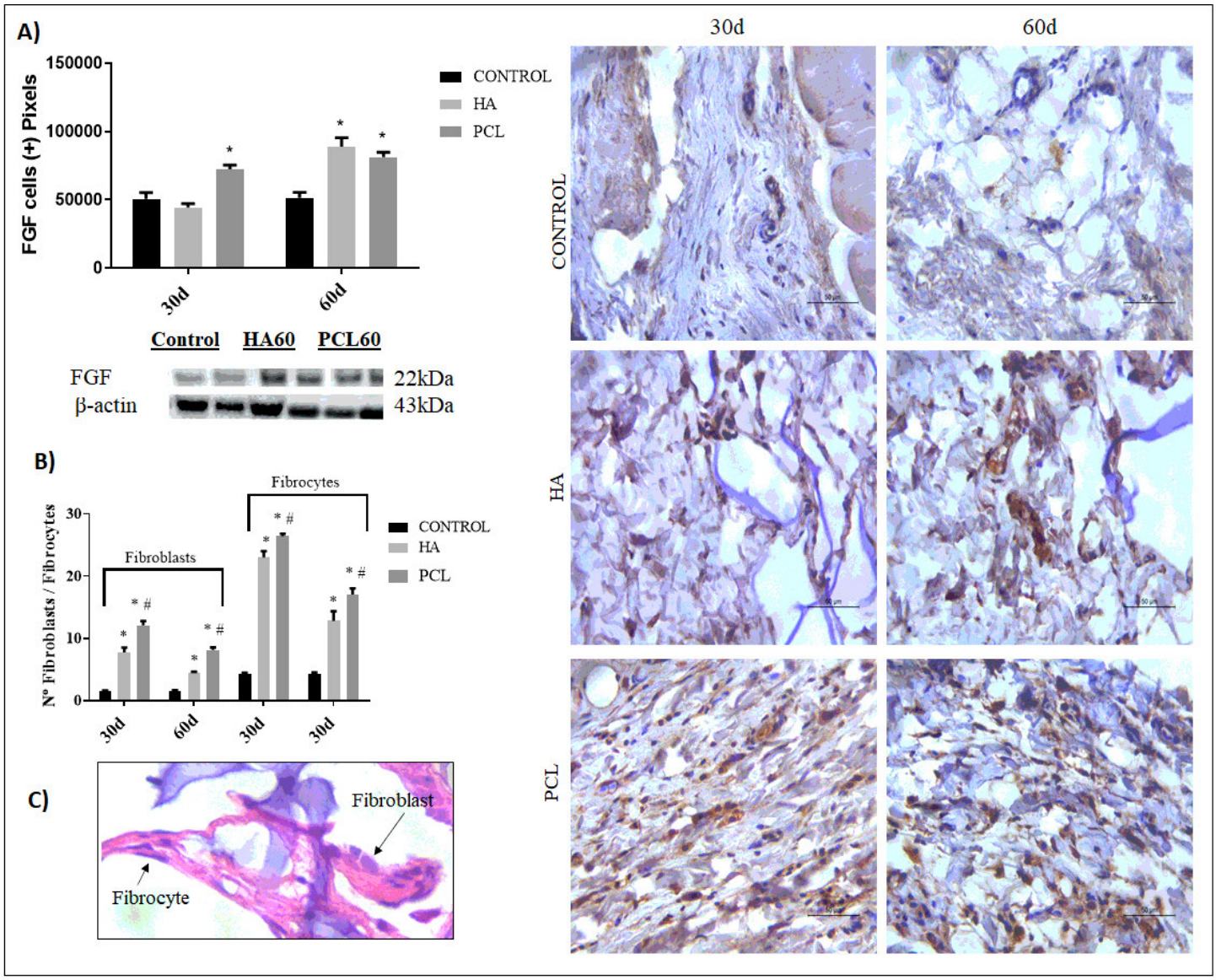

Figure 3

Number of fibroblasts / fibrocytes, immunostaining and FGF protein expression.

A. Immunostaining for FGF at 30 and 60 days and quantification of 1 expressed in the graph. **B.**Fibrocyte and fibroblast count at 30 and 60 days represented in the graph. **C.**Illustrative image of the fibrocyte and fibroblast in the connective tissue adjacent to the biomaterial. Bars show mean ± SEM of 6 animals per group. * represents the significant difference of HA and PCL when compared to CONTROL, at 30 and 60

days. # represents the significant difference between PCL and HA. (*p< 0.001), ANOVA followed by Tukey's test).

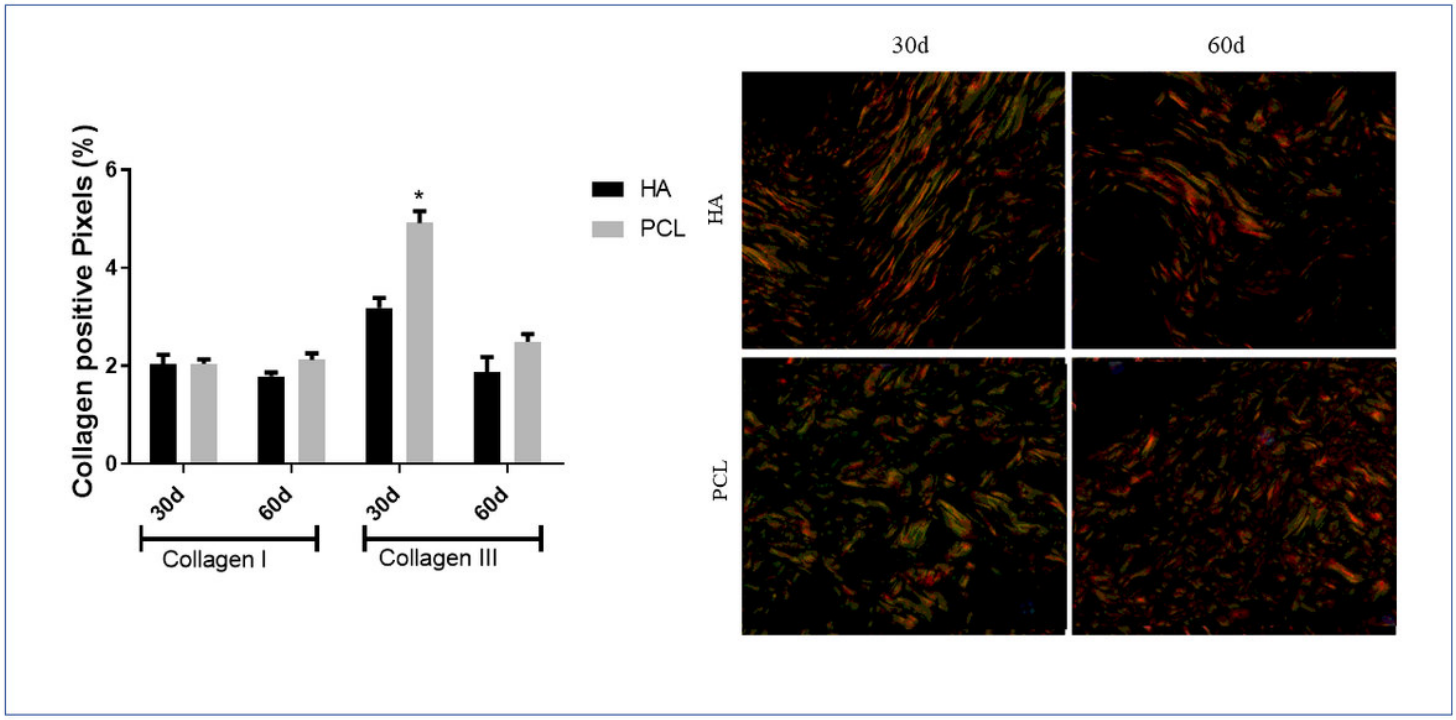

Figure 4

Graph representing the quantification of type I and III collagen fibers stained with Picrosirius Red and photomicrography of the skin of rats in the HA and PCL experimental groups. * Represents a significant difference in PCL compared to HA at 30 days. (*p< 0.001), ANOVA followed by Tukey's test. It is possible to observe the presence of collagen type I (red) and III (green). 400x magnification.