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Pró-Reitoria de Pesquisa e Pós-Graduação
Centro de Ciências Biológicas e da Saúde
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Doutorado

HEPATITE B (VHB) E DELTA (VHD) EM CINCO MUNICÍPIOS DO ESTADO MARANHÃO: prevalência e fatores associados

JOMAR DIOGO COSTA NUNES

São Luís
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Orientadora: Profa. Dra. Adalgisa de Souza Paiva Ferreira

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Aprovado em: _____ / _____ / _____

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Todas as vitórias ocultam uma abdicação”

Simone de Beauvoir

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RESUMO

Introdução: A infecção pelo vírus da hepatite B (VHB) pode se tornar crônica. A co-infecção com o vírus Delta (VHD) pode piorar a primeira, favorecendo maior risco para cirrose hepática e carcinoma hepatocelular (CHC). No Brasil, as taxas de prevalência destas infecções são heterogêneas. O objetivo desse estudo foi estimar as prevalências das hepatites B e Delta em cinco municípios localizado no estado do Maranhão no Brasil (Axixá, Morros, Icatu, Humberto de Campos e Urbano Santos). **Metodologia**: A amostra foi calculada, levando em consideração uma prevalência de 0,5% do HBsAg (considerando o resultado da prevalência do HBsAg, encontrada no inquérito nacional na região Nordeste) com erro absoluto de 0,3%, nível de confiança de 95% com uma amostra total de 3.856 a 4.000 indivíduos maiores de 1 ano de idade. Foram aplicados questionários para identificar características sóciodemográficas e fatores de risco de transmissão. Amostras de sangue foram coletadas e foram realizadas sorologias para HBsAg, anti-HBc, anti-HBs e anti-HDV por ensaio imunoenzimático (ELISA). **Resultado:** Um total de 3984 participantes foram incluídos. A prevalência de HBsAg foi de 2,30%, 38,47% tinham anti-HBc positivo,13,95% tinham anti-HBc isolado e 20,10% apresentaram anti-HBs isoladamente positivo. A prevalência de VHD na amostra total foi de 0,35% (entre os 92 portadores do VHB, oito tinham o marcador anti-HDV positivo, correspondendo a 8.6%). **Conclusão**: A prevalência do HBsAg na região foi considerada intermediária (mais de 2% da população é portadora) e entre estes, 8% tinham positividade para o VHD, sugerindo que é uma região em que medidas rigorosas de prevenção devem ser implementadas para prevenir a infecção por estes vírus impedindo a progressão para cirrose hepática e CHC.

Palavras-chave: Hepatite B; Hepatite Delta; prevalência; fatores associados

ABSTRACT

Introduction. Hepatitis B Virus (HBV) infection can lead to chronic disease. Hepatitis Delta virus (HDV) coinfection may favor an increased risk for liver cirrhosis and hepatocellular carcinoma (HCC) in HBV carriers. HBV infected individuals may present detectable HBV DNA with absence of hepatitis B surface antigen (HBsAg). HBV and HDV infections prevalence rates are heterogeneous in Brazil. The aim of this study was to estimate the prevalence of HBV and HDV infections located in the state of Maranhão in Brazil (Axixá, Morros, Icatu, Humberto de Campos and Urbano Santos). **Methods**. A total of 3.856 to 4,000 individuals participated of this study. Sample size was obtained by considering a prevalence of 0.5% of HBsAg (according to previous national survey), absolute error of 0.3% and 95% confidence interval. Individuals should be age 1 or older to participate in this study. Questionnaires were applied to identify sociodemographic characteristics and transmission risk factors. Samples of blood were collected and serologies were performed for HBsAg, anti-HBc, anti-HBs and anti-HDV markers by enzyme-linked immunosorbent assay (ELISA). **Results**. A total of 3.984 individuals were screened for HBsAg serological markers. The prevalence of HBsAg was 2.30%. Anti-HBc marker was found in 38.47% of samples, 13.95% and 20.10% presented isolated anti-HBc and anti-HBs respectively. Considering the 3,984 samples, anti-HDV was found in 0.35% individuals (among the 92 HBV carriers, eight presented the anti-HDV marker, corresponding to 8.6%). Among the 561 participants with anti-HBc alone, 223 (39.75%) **Conclusion**. The prevalence of HBV in the region was considered to be intermediate (more than 2% of carriers). Among those carriers, 8% tested positive for anti-HDV. Thus, prevention measures should be implemented to prevent Infection by these viruses and progression to liver cirrhosis and HCC.

Keywords: hepatitis B; hepatitis delta; prevalence; associated factors

LISTA DE SIGLAS E ABREVIATURAS

Anti-HBc	Anticorpos contra o core do vírus da Hepatite B
Anti-HBs	Anticorpo contra o antígeno de superfície do vírus da Hepatite B
Anti-HBe	Anticorpo contra o antígeno HBe
Anti-HDV	Anticorpo contra o vírus da hepatite Delta
ALT/TGP	Alanina aminotransferase/transaminase glutâmico-pirúvica
AST/TGO	Aspartato aminotransferase/transaminase glutâmico-oxalacética
CHC	Carcinoma hepatocelular
ELISA	Ensaio de imunoabsorção enzimática
HBcAg	Antígeno do core do vírus da Hepatite B
HBeAg	Antígeno HBe
HBsAg	Antígeno de superfície da Hepatite B
HBV	Vírus da Hepatite B
HCV	Vírus da hepatite C
HDV	Vírus da hepatite D
IST	Infecções Sexualmente Transmissíveis
SIDA	Síndrome da Imunodeficiência Humana Adquirida
PCR	Polymerase Chain Reaction

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SUMÁRIO

1. Introdução

A infecção pelo vírus da hepatite B (VHB) é a mais comum infecção viral crônica no mundo, com cerca de 257 milhões de pessoas cronicamente infectadas, sendo responsável por, aproximadamente, 887.000 mortes anualmente. Esses dados levaram a Organização Mundial de Saúde (OMS) a incluí-la em suas principais prioridades de saúde pública (WHO, 2017).

A mesma OMS classifica a endemicidade da hepatite B, de acordo com a prevalência do marcador sorológico antígeno de superfície da hepatite B (HBsAg), em endemicidade baixa (< 2%), intermediária baixa (2% a 4%), intermediária alta (5% a 7%) e alta (≥ 8%) (WHO, 2017).

O Brasil é classificado como uma região de intermediária endemicidade, com uma distribuição heterogênea da doença. Entretanto, estudo patrocinado pelo Ministério da Saúde, que avaliou quase 20.000 pessoas com mais de 9.000 famílias nas 27 capitais brasileiras de 2004 a 2009, colocou o país em uma classificação de baixa endemicidade. Esse declínio poderia ser considerado resultado de melhorias socioeconômicas em uma parcela da população e a cobertura efetiva de vacinação. Porém, esses achados são controversos já que a amostra representou apenas os centros urbanos, excluindo as regiões mais pobres e com piores índices de saúde e condições econômicas, provavelmente subestimando a verdadeira prevalência da hepatite B (PEREIRA et al., 2009; XIMENES et al., 2015).

Em portadores do VHB, se há co-infecção com o vírus da hepatite Delta (VHD), a doença hepática pode ser de maior gravidade e evoluir com maior frequência para as complicações tais como a cirrose hepática e o carcinoma hepatocelular (CHC). Não há estudos definitivos sobre a prevalência do VHD no Brasil, mas sabe-se que as maiores taxas estão na Amazônia Ocidental (NOUREDDIN e GISH, 2014; GIERSCH et al., 2014)

No ambulatório de doenças hepáticas do Hospital Universitário da Universidade Federal do Maranhão, tem sido observada uma frequência elevada de indivíduos portadores do VHB procedentes dos municípios maranhenses de Humberto de Campos, Urbano Santos, Axixá, Morros e Icatu. Nessa mesma população foram identificados portadores da co-infecção VHB-VHD (BARROS et al.,

2011). Tais achados motivaram a realização de um grande estudo envolvendo a população daqueles municípios, para identificar a estimação da prevalência destas infecções, os fatores de risco associados e os genótipos virais lá identificados.

O estudo intitulado “Prevalência das Hepatites B, C e D nos municípios de Urbano Santos e da Região do Baixo Munin, Maranhão, Brasil”, já resultou em três artigos recentemente publicados: o primeiro artigo sobre genótipos do VHD, intitulado: *“The hepatitis delta genotype 8 in Northeast Brazil: The North Atlantic slave trade as the potential route for infection*” (SANTOS et al., 2016); o segundo sobre perfil sorologico anômalo do VHB intitulado: “*Mutation in the a-determinant of the S gene of the hepatitis B virus associated with concomitant HBsAg and anti-HBs in a population in northeastern Brazil*” (DE CAMPOS et al., 2017); e o terceiro sobre genótipos do VHB intitulado: *High prevalence of Hepatitis B subgenotype D4 in Northeast Brazil: an ancient relic from African Continent?* (SANTOS et al., 2018). O presente estudo apresenta os resultados finais das prevalências das infecções pelo VHB e VHD e os fatores de risco associados à infecção.

2. Referencial teórico

2.1 Hepatite B

2.1.2 Vírus B

O vírus da hepatite B (VHB) é um pequeno vírus de DNA, da família *Hepadnaviridae,* gênero *Hepadnavírus,* com aproximadamente 42-47 nm de diâmetro com características semelhantes a retrovírus, cuja replicação ocorre através de um intermediário de RNA e pode integrar-se no genoma do hospedeiro, essa necessidade de transcrição reversa para a replicação do genoma do DNA acarreta mutações virais frequentes nos genomas (BLOCK et al., 2007; LIANG, 2009; LÜSEBRINK et al., 2009; KARAYIANNIS, 2017) . Assim como os demais hepadnavirus, o VHB tem predileção pelos hepatócitos. Partículas virais podem ser encontradas também em tecidos extra-hepáticos como baço, coração, pâncreas, medula óssea e rins (KIDD-LJUNGGREN, 2002; LOCARNINI et al., 2003).

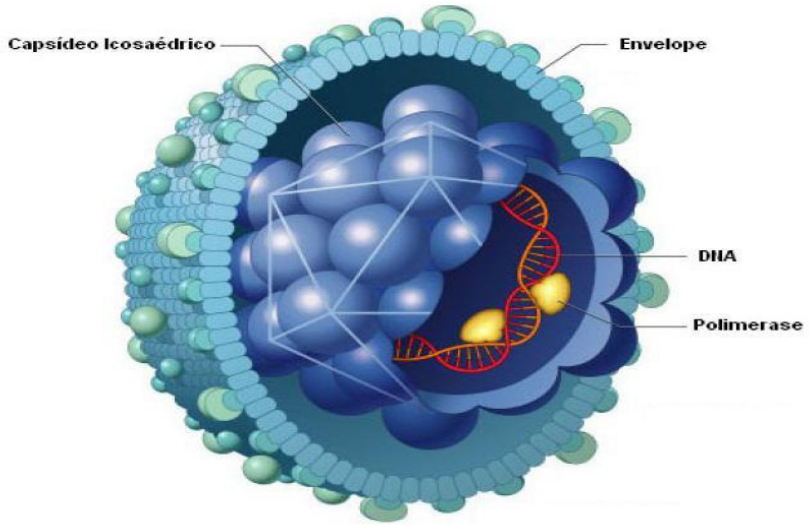

Figura 1- Representação esquemática da estrutura do Vírus da Hepatite B.
Fonte: http://people.rit.edu/japfaa/index.html -
[Figura adaptada de Perkins para o português].

A partícula viral completa (vírion) do VHB é uma estrutura esférica chamada partícula de Dane (DANE et al., 1970) (Figura 1). É constituída por uma camada externa lipídica que contém o antígeno de superfície (HBsAg), envolvendo um nucleocapside central (HBcAg), que contém o genoma viral e a enzima DNA-polimerase (LEE e AHN, 2011; VALAYDON e LOCARNINI, 2017).

Na estrutura também se encontra o HBeAg, antígeno solúvel, que está relacionado com a replicação viral. É um vírus extremamente resistente e capaz de suportar altas temperatura e umidade; pode sobreviver fora do organismo hospedeiro por um período longo, por 15 anos quando conservado em temperatura de 20ºC, 6 meses em temperatura ambiente e 7 dias em temperatura de 44º C (LOCARNINI, 2003; LOK e MCMAHON, 2007).

2.1.3 Prevalência da hepatite B

A infecção pelo VHB é um expressivo problema de saúde pública no mundo, com amplo impacto econômico e social, uma vez que mais de dois bilhões de indivíduos vivos, o equivalente a um terço da população mundial, já foram infectados pelo VHB em algum momento de suas vidas e cerca de 257 milhões permanecem cronicamente infectados (WHO, 2017).

A Organização Mundial de Saúde (OMS) classifica a endemicidade da hepatite B, de acordo com a prevalência HBsAg, em endemicidade baixa (< 2%), intermediária baixa (2% a 4%), intermediária alta (4% a 7%) e alta (≥ 8%) (WHO, 2017).

Entre os portadores crônicos do VHB, 75% vivem nas regiões Ásia-Pacifico (incluindo China, Japão, Korea, Filipinas e Vietnam) e África Subsaariana (MACLACHLAN et al., 2015). O restante do mundo, incluindo a maioria da Europa e Américas é considerado ser de baixa prevalência (SCHWEITZER et al., 2015; CHANG e NGUYEN, 2017). Nestas últimas regiões, esta pode ser maior entre imigrantes provenientes de países de alta ou intermediária prevalência e entre pessoas incluídas em grupos de alto risco (FALLA et al., 2018).

Um dos motivos para as diferentes taxas de prevalência identificadas no mundo está provavelmente relacionado com as diferenças de idade em que a infecção é adquirida (fator que determina a taxa de cronificação) (WU e CHANG, 2015). O VHB acomete todas as faixas etárias, porém o risco de os pacientes tornarem-se portadores crônicos é inversamente proporcional à idade em que se contaminam. Entre os recém-nascidos, mais de 90% tornam-se cronicamente infectados. Essa possibilidade cai para 30 a 50%, se a infecção for até os 5 anos, sendo que nos adultos o risco é menor que 5% (LOK, 2016).

Em todo o mundo, as taxas de endemicidade permanecem caindo nas últimas décadas devido a implementação dos programas de vacinação contra o vírus (NAYAGAM et al., 2016; OTT et al., 2017).

O Brasil tem sido considerado, desde 2015 (SCHWEITZER et al., 2015), como sendo de baixa endemicidade (menos de 2%), devido ao resultado de um estudo de base populacional que envolveu todas as capitais brasileiras, que identificou soro-prevalência do HBsAg de 0.63%, 0.48%, 0.37%, 0.31%, 0.31% e 0.26% respectivamente nas regiões Norte, Sul, Nordeste, Centro-Oeste, Sudeste e Distrito Federal (PEREIRA et al., 2009; XIMENES et al., 2015).

Este inquérito apresenta limitações, alegadas pelos próprios pesquisadores, por incluir apenas as capitais, provavelmente subestimando a real prevalência da hepatite B no país. Souto (2016), fazendo uma revisão sistemática dos últimos estudos de prevalência publicados, identificou que, de fato, ocorre heterogeneidade na sua distribuição variando de endemicidade intermediária alta (4 a 7%) como observada em regiões da Amazônia ocidental, intermediária baixa (2 a 4%) identificada em áreas rurais do Mato Grosso e Pará, e o restante do país apresentando resultados abaixo de 2%. Na mesma revisão de Souto, observou-se que nos estudos anteriores a 2006, as taxas de prevalência, especialmente na região amazônica, podiam chegar até 10% de endemicidade.

É possível que esta queda ao longo da última década esteja relacionada com o uso em larga escala da vacina contra a hepatite B desde 1998, à baixa frequência de usuários de drogas ilícitas injetáveis e a medidas educativas frequentes dirigidas entre os jovens sobre a síndrome da imunodeficiência adquirida (SIDA) (MELO et al., 2015).

No Maranhão alguns estudos demonstraram a endemicidade do HBsAg de 0,9% em gestantes atendidas em maternidades públicas de São Luís (SOUZA et al., 2012) e de 12,5% em descendentes de quilombolas no município de Mirinzal, que viviam em comunidades isoladas (ALVARADO-MORA et al., 2011). Ainda, a taxa de 0,6% foi identificada em doadores de sangue no Estado do Maranhão (VIANA et al., 2009) e 2,88% em um município na zona rural de Mirinzal (EL KHOURI et al., 2010).

2.1.4 Transmissão do VHB

O VHB pode ser encontrado no sangue e em algumas secreções corporais em menor concentração (líquido seminal e secreções vaginais) (MARINHO e MACEDO, 2009).

A transmissão do vírus pode ocorrer pelas vias perinatal (durante a gestação e/ou na passagem pelo canal do parto), parenteral/percutânea (compartilhamento de agulhas e seringas em usuários de drogas injetáveis, acidentes ocupacionais, tatuagens, colocação de *piercings*, acupuntura, procedimentos odontológicos ou cirúrgicos e hemodiálise) e pela via sexual, sendo considerada uma infecção sexualmente transmissível (TE e JENSEN, 2010; SILVA et al., 2012). A outra via de transmissão do vírus é a horizontal, que pode ocorrer por contato contínuo com familiares portadores, principalmente nos primeiros anos de vida entre as crianças (WASMUTH, 2009; MELO e ISOLANI, 2011).

A transmissão perinatal é mais frequente nas regiões de alta endemicidade, onde mulheres contaminadas na infância mantém replicação viral durante a gravidez, transmitindo a infecção para seus filhos e perpetuando um ciclo vicioso de manutenção da infecção (LIVINGSTON et al., 2007; ZAMPINO et al., 2015; YI et al., 2016). A transmissão horizontal é também observada em regiões de endemicidades alta, mas também em regiões de prevalência intermediária, onde a contaminação ocorre após o nascimento, como acontece na Amazônia ocidental (WASMUTH, 2009). Em regiões de baixa prevalência (como os da Europa Ocidental e América do Norte), a maioria das infecções é transmitida durante a vida adulta pela atividade sexual e uso de drogas injetáveis (WHO, 2017).

Outro grupo de risco são os profissionais de saúde, os quais estão susceptíveis a acidentes de trabalho com exposição a material biológico (LEWIS et al., 2015). Dentre os acidentes, destaca-se o descarte inadequado de materiais perfuro cortantes, o que expõe os diversos profissionais, inclusive do serviço de limpeza e de coleta de resíduos, ao risco de acidentes e a exposição a materiais biológicos contaminados (JULIO et al., 2014). Apesar da imunidade duradoura e específica conferida à hepatite B, observa-se um esquema vacinal incompleto em profissionais de saúde (CUNHA et al., 2016; REAM et al., 2016), demonstrando a

necessidade da concientização dessa população para uma melhor implementação dos programas de prevenção da hepatite B (MARINHO et al., 2014).

Em relação ao comportamento sexual, observa-se que homens que fazem sexo com homens apresentam maiores taxas de marcadores de infecção prévia pelo VHB, onde os fatores de risco são: idade supeior aos 25 anos; ser passivo; sexo anterior com as mulheres; e história de infecções por infecções sexualmente transmissíveis (IST) (OLIVEIRA et al., 2016). Essa maior vulnerabilidade envolve o contexto da violência, condições de práticas sexuais, como sexo anal desprotegido e múltiplos parceiros sexuais, além de baixo acesso aos serviços de saúde e integração social, o que pode levar a práticas sexuais inseguras (OLIVEIRA et al., 2016). Na mesma linha anterior, os profissionais do sexo apresentam uma prevalência da hepatite B maior de que a populção em geral (SCHUELTER-TREVISOL et al., 2013).

2.1.5 Diagnóstico da infecção pelo VHB

O diagnóstico sorológico da hepatite B é realizado através da identificação do antígeno de superfície do VHB (HBsAg) e seu anticorpo (anti-HBs), do anticorpo produzido em resposta ao antigeno do core (anti-HBc: frações IgG e IgM), do antígenio do envelope (HBeAg) e seu anticorpo (anti-HBe) (HOLLINGER e SOOD, 2010). Esses marcadores sorológicos são utilizados para o diagnóstico de diferentes fases da infecção, onde a diferença entre as fases aguda, crônica e de resolução (espontânea ou não) ocorre através da identificação do HBsAg, anti-HBc e anti-HBs. Enquanto o HBeAg e o anti-HBe são utilizados normalmente como exame de acompanhamento de pacientes com infecção crônica (MAST et al., 2005; ALLAIN e Opare-Sem, 2016). Na história natural da infecção por VHB ocorrem com padrões sorológicos característicos (Quadro 1).

Quadro 1: Interpretação dos resultados do teste sorológico de hepatite B

Marcadores sorológicos					
HBsAg	Anti-HBc-Total	Anti-HBc (IgM)	Anti-HBs	HBeAg	Interpretação
Negativo	Negativo	Negativo	Negativo	Negativo	Sem contato prévio com o vírus
Negativo	Negativo	Negativo	Positivo	Negativo	Imunizado após vacinação
Negativo	Positivo	Negativo	Positivo	Negativo	Imunizado após contato com o vírus
Positivo	Positivo	Positivo	Negativo	Positivo	Infecção aguda
Negativo	Positivo	Positivo	Positivo ou Negativo	Negativo	Resolução da infecção aguda
Positivo	Positivo	Negativo	Negativo	Positivo	Infecção crônica com alta replicação
Positivo	Positivo	Negativo	Negativo	Negativo	Infecção crônica com baixa ou alta replicação
Negativo	Positivo	Negativo	Negativo	Negativo	antiHBc isolado (pode ser infecção oculta)

Para a identificação destes marcadores sorológicos, realiza-se ensaios imunoenzimáticos (ELISA) e baseia-se na interação entre epitopos das proteínas virais e seus respectivos anticorpos específicos (CHAKRAVARTY, 2011). No tecido hepático o HBsAg e o HBcAg podem ser detectados por meio de técnicas moleculares, como hibridização *in situ*. (COLLIER e OXFORD, 2000).

Além do diagnóstico sorológico, é importante a realização de testes moleculares para detecção da carga viral. O teste mais utilizado atualmente é a Reação em Cadeia da Polimerase – (PCR) em tempo real que identifica o genoma viral, que pode ser quantificado (carga viral do VHB-DNA, descrito em UI/ml) (LIU et al., 2006; SITNIK et al., 2010; GUIRGIS et al., 2010).

A carga viral é fundamental para o diagnóstico e acompanhamento dos pacientes com o VHB. Portadores crônicos com replicação viral caracterizam-se por um nível de VHB-DNA> 2000 UI / mL e apresentam um risco elevado de progressão para cirrose e carcinoma hepatocelular, na ausência de tratamento anti-viral (LIN et al.,2016). Enquanto portadores com níveis de DNA permanentemente abaixo de 2000 UI / mL, tem um melhor prognóstico na ausência de tratamento (ILOEJE et al., 2012).

Outros testes importantes que contribuem no diagnóstico da doença são as aminotransferases (ALT/TGP alanina aminotransferase/transaminase glutâmico-

pirúvica e AST/TGO aspartato aminotransferase/transaminase glutâmico-oxalacética). Embora não sejam específicas para nenhum tipo de hepatite, estas enzimas são indicadores sensíveis e marcam sinais de atividade necroinflamatória hepática e podem ser importantes na definição da fase da infecção (WHO, 2017).

2.1.6 Genótipos Virais

O genoma do VHB apresenta diversidade na sua sequência de nucleotídeos que permite sua classificação em genótipos. Atualmente existem já identificados 10 genótipos, classificados de A-J (SUNBUL, 2014; YU et. al., 2010; AGARWAL et al., 2015).

Os genótipos têm distribuição geográfica variada e aspecto epidemiológico, sua heterogeneidade parece estar relacionada com diferenças na evolução clínica da infecção e na resposta ao tratamento antiviral. Cada genótipo é caracterizado por uma distribuição étnica e geográfica distinta, em todo o mundo (POURKARIM et al., 2014). O genótipo A é prevalente no noroeste da Europa, América do Norte e Africa. Os genótipos B e C são comumente encontrados na Asia, ao passo que o genótipo D mostra uma distribuição mundial, mas predomina na região do Mediterrâneo, incluindo o Oriente Médio e centro da Ásia. O Genótipo E é encontrado na região ocidental da Africa, o genótipo F está presente na população indígena da América do Sul, e o genótipo H é encontrado na população indígena da América Central (NORDER et al., 2004; CROAGH et al., 2015).O genótipo G foi inicialmente isolado em portadores de VHB na França e na Geórgia (Estados Unidos) e depois foi detectada na Inglaterra, Itália e Alemanha. Recentemente o genótipo I do VHB foi descrito no noroeste da China, Vietnã e Laos (YU et al., 2010; MELLO et al., 2014).

No Brasil predomina o genótipo A seguido pelos genótipos D e F (LAMPE et al.,2017). As regiões com maior frequência do genótipo A, são as Regiões Norte, Nordeste e Sudeste, enquanto o genótipo D é mais frequente na Região Sul e o F na Amazônia (MELLO et al., 2007). Os genótipos B e C também já foram identificados no Brasil em baixa prevalência, provavelmente refletindo o baixo percentual de descendentes asiáticos nos estados brasileiros (SITNIK et al., 2004).

No Maranhão, estudo populacional em serviço especializado identificou os genótipos mais prevalentes o A (67%), D (28%) e F (5%) (BARROS et al. 2014). Entretanto, quando avaliada a associação do VHB com o virus da hepatite Delta (VHD) o mais frequente é o genótipo D, seguido pelo genótipo A (BARROS et al., 2011; SANTOS et al., 2016).

A presença destes genótipos reflete a diversidade de etnias no Brasil: Índio americano, Europeu e Africano, raízes ancestrais mostrando o país como um importante modelo para estudos de genética de populações, sugerindo uma influência do padrão de imigração para cada região (ALCALDE et al., 2009).

2.1.7 História Natural da Infecção

Uma vez que o indivíduo contaminado pelo VHB pode eliminar o vírus ou torna-se portador crônico, a definição de infecção crônica é dada pela persistência da positividade do HBsAg por mais de seis meses (HOOFNAGLE et al., 2007).

A possibilidade da infecção tornar-se crônica está diretamente relacionada com a idade do contágio e o estado de replicação do vírus no transmissor: As crianças infectadas através da via perinatal, cujas mães apresentam HBeAg positivo, têm mais de 90% de chance de se tornarem portadoras crônicas; este risco cai para 30%, se a mãe for anti-HBe positivo. Se a infecção ocorrer entre o nascimento e os dois anos de idade, a taxa de cronificação chega a 50%, caindo a menos de 10% se a infecção ocorrer após os 10 anos (MCMAHON, 2010).

Estudos demonstram que 15 a 40% dos indivíduos, que se tornam portadores crônicos, podem evoluir para cirrose hepática e/ou carcinoma hepatocelular (LAVANCHY, 2004; SHEPARD et al., 2006; BOTTECCHIA et al., 2011).

Uma vez portador crônico, o indivíduo pode passar pelas seguintes fases:

(1) Fase de tolerância imunológica: ocorre em geral de forma prolongada, quando a transmissão é perinatal ou horizontal na infância e pode durar até a quarta década da vida. Caracteriza-se pela positividade do HBeAg, elevada replicação viral e ausência de qualquer sinal de agressão ao fígado (aminotransferases e histologia hepática normais)

(2) Fase Imunoativa: que geralmente se constitui também na primeira fase da infecção crônica, quando o vírus é transmitido de outra forma que não às descritas na fase anterior. Aqui, já há reconhecimento do vírus pelo sistema imunológico, com agressão ao fígado (elevação das aminotransferases e histologia com inflamação e fibrose), o HBeAg pode ser positivo ou negativo e a carga viral é mais baixa. Quanto maior a duração desta fase, maior a probabilidade de evoluir para cirrose hepática.

(3) Fase inativa: quando há a soroconversão HBeAg/anti-HBe, com carga viral negativa ou muito baixa e aminotransferases normais. Esta fase pode durar por toda a vida do indivíduo, sem evolução para cirrose hepática ou pode apresentar fases de reativação da doença semelhante à anterior.

(4) Fase de resolução: quando o portador crônico elimina espontaneamente o HBsAg, o que pode ocorrer em cerca de 0,5 a 0,8% a cada ano. Nesta fase ocorre a parada de progressão da doença inflamatória do fígado, mas ainda permanece o risco de desenvolver HCC.

Além das formas de transmissão, outros fatores relacionados tanto ao vírus como ao hospedeiro, podem contribuir para o comportamento da infecção crônica, tais como, genótipo do VHB, co-infecção com outro vírus, estado imunológico do hospedeiro, gênero, ingestão alcoólica e fatores genéticos (PUNGPAPONG et al., 2007; HADZIYANNIS, 2011).

Mais recentemente tem sido sugerida uma nova classificação para a infecção crônica pelo VHB, que seria dividido em fases (Quadro 2), não necessariamente sequenciais, levando-se em conta a presença do HBeAg, concentração de HBVDNA, os valores da ALT e a presença ou ausência de inflamação hepática. A nova nomenclatura baseia-se na descrição de duas principais características de cronicidade: infecção e hepatite (EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER, 2017).

Quadro 2: História natural da infecção crônica pelo VHB

	HBeAg positivo		HBeAg negativo	
	Infecção crônica	**Hepatite crônica**	**Infecção crônica**	**Hepatite crônica**
HBeAg	Positivo	Positivo	Negativo	Negativo
HBVDNA	> 10^7 UI/mL	10^4-10^7 UI/mL	< 2000 UI/mL	> 2000 UI/mL
ALT	Normal	Elevada	Normal	Elevada
Inflamação histológica	Ausente ou mínima	Moderada ou Severa	Ausente	Moderada ou Severa
Nomenclatura antiga	Imunotolerante	Imunoativo	Portador inativo	Reativação

HBeAg: antígeno e do vírus B; HBVDNA: DNA do vírus B; ALT: alanina aminotransferase
Fonte: adaptada de European Association for the Study of the Liver. EASL 2017

2.2 Hepatite Delta

Na década de 1970, foi identificado o vírus da hepatite Delta (VHD) em áreas endêmicas de hepatite B no sul da Europa (RIZZETTO et al., 1977), desde então sua infecção tem sofrido nitidamente uma redução em todo o mundo, levando a se prever que no início do século 21 esse vírus seria erradicado, porém esse fato não se concretizou (RIZZETTO e CIANCIO, 2012).

Os mecanismos de transmissão do VHD são os mesmos do VHB, preferencialmente por via parenteral, podendo ser na forma de superinfecção (infecção pelo Delta em portadores da infecção pelo VHB) ou co-infecção, quando os dois vírus são transmitidos juntos (RIZZETTO, 2014).

Para que ocorra a fixação e entrada do VHD nos hepatócitos há necessidade da presença do HBsAg, porém essa dependência é exclusivamente para a construção do envelope viral, sendo evidenciada que sua infecção pode persistir mesmo na ausência de replicação do VHB (ou quando a replicação está profundamente suprimida) (FREITAS et al., 2014). Tal vírus pode ser responsável por hepatite fulminante em infecções agudas e quando presente em portadores crônicos do VHB possibilita uma progressão acelerada da doença hepática, com maiores taxas de cirrose e de carcinoma hepatocelular (CHC) (NOUREDDIN e GISH, 2014; GIERSCH et al., 2014). Cerca de 70-90% dos casos de hepatite delta crônica desenvolve cirrose (SINGH et al., 2012). Esse risco é potencializado quando

o indivíduo apresenta replicação concomitante do VHB e também co-infecção com o vírus da hepatite C (HCV) (BAHDE et al., 2011; GISH et al., 2013).

O VHD tem distribuição geográfica heterogênea, estimando-se cerca de 18 milhões de infectados entre os 250 milhões de portadores crônicos do VHB, ou seja, mais de 5% deles também apresentam infecção pelo VHD (RIZZETTO, 2015; WRANKE et al., 2018).

Nos anos 1980 havia elevada endemicidade do VHD em algumas regiões do mundo, como bacia do Mediterrâneo, Oriente Médio, Ásia Central e do Norte, África Ocidental e Central, bacia Amazônica (Brasil, Peru, Venezuela e Colômbia) e ilhas do Pacífico (ALVARADO-MORA e PINHO, 2013). Com a implementação universal dos programas de vacinação para o VHB, o VHD deixou de ser endêmico na maioria dos países industrializados, mas ainda é um grave problema na bacia Amazônica e na África (KONDILI et al., 2010, RIZZETTO e CIANCIO, 2012; RIZZETTO, 2016).

Na Europa o VHD ainda tem sido associado a imigrantes e/ou portadores do HIV, como observado nos estudos de Rivas et al. (2013), mas também observa-se eventualmente a presença apenas de casos esporádicos (RIZZETTO e CIANCIO, 2012).

No Brasil a maior prevalência do VHD ocorre na Amazônica Ocidental, sendo ainda responsável por casos de hepatite fulminante (GOMES-GOUVÊA et al., 2009) e importante causa de doença hepática crônica naquela região (CICERO et al., 2016).

Têm sido observados casos fora da região Amazônica como em São Paulo e no Maranhão (STRAUSS et al., 1987; BARROS et al., 2011; MENDES-CORREA et al., 2011).

Esse vírus possui oito diferentes genótipos variando do 1 ao 8, onde: o VHD-1 apresenta ampla distribuição mundial sendo ainda o mais frequente, destacando-se na Europa, Oriente Médio, América do Norte e Norte da África; o VHD-2 é observado no leste asiático; o VHD-3 é mais prevalente na bacia Amazônica; o VHD-4 presente apenas nas ilhas Miyako em Okinawa, no Japão; enquanto os genótipos 5 ao 8 encontram-se predominantemente na África (WEDEMEYER e MANNS, 2010; LE GAL et al., 2017).

Apesar de o VHD necessitar do HBsAg para sua replicação (ABBAS; SIDDIQUI, 2011), há uma tendência de que a presença do VHD suprima a replicação do VHB, tornando a carga viral média do VHB significativamente menor comparando-se com a mono-infecção, mas com um aumento da carga viral do VHD com a progressão da doença (KIESSLICH et al., 2009).

A quantificação do DNA do VHD em diferentes fases da infecção é importante para o diagnóstico. Várias técnicas *in house* e comerciais têm sido descritas para a quantificação do RNA do VHD, porém tais metodologias diferem amplamente, não existindo normas internacionais padronizadas para a quantificação do VHD, sendo necessária a realização de estudos interlaboratoriais com o intuito de estabelecer padrões generalizados (HOMS et al., 2014).

Técnicas quantitativas para o VHD apresentam como principal dificuldade a estabilização do RNA. Observa-se ainda que as tentativas de padronizações de metodologia quantitativas para o VHD ocorrem principalmente para o genótipo 1, dificultando sua aplicação de forma generalizada (KARATAYL et al., 2014).

O estudo de Brichler et al. (2013) analisou metodologias *in house* e três testes comerciais e observou que os testes comerciais apresentavam sensibilidade apenas para o genótipo 1 em amostras europeias ou asiáticas, sendo os seus resultados inadequados para amostras africanas e com genótipos diferentes do 1. Esse resultado foi semelhante ao estudo Shang et al. (2012), que demonstrou que a técnica *in house* RT-*PCR (reação de transcriptase reversa, seguida de reação de cadeia polimerase)* é capaz de detecção de todos os genótipos conhecidos VHD (SHANG et al., 2012).

Recentemente foi desenvolvido um teste por pesquisadores brasileiros na Amazônia, que se mostrou bastante sensível (BOTELHO-SOUZA et al., 2014).

É necessário destacar que a ocorrência da infecção pelo VHD em muitas regiões do mundo pode estar subnotificada, principalmente pela escassez da realização de teste de anti-VHD (RIZZETTO e CIANCIO, 2012). Dessa forma, torna-se necessário para a expansão do diagnóstico de hepatite Delta, incluir nos protocolos exames de triagem para o VHD em todos os portadores do VHB.

Essa necessidade da expansão dos exames de triagem foi confirmada nos estudos em doadores de sangue realizados na França, onde os portadores do

VHB foram triados para o VHD, encontrando-se uma frequência de 6,5%, sendo os genótipos mais prevalentes: VHD-1, VHD-6 e VHD-7, os dois últimos atribuídos à imigração, pois habitualmente são genótipos de países africanos (SERVANT-DELMAS et al., 2014).

Estudo realizado por nosso grupo também identificou casos do VHD no Maranhão pela primeira vez. Em uma amostra de portadores crônicos do VHB foram identificados três pacientes portadores desse vírus, um paciente com VHD-3 (nascido na Amazônica Ocidental) e surpreendentemente dois pacientes infectados com VHD-8, um genótipo africano. Esses pacientes nasceram e sempre viveram em Urbano Santos, município rural do estado do Maranhão. Além disso, foi a primeira descrição do VHD-8 em populações africanas não nativas (BARROS et al., 2011).

3. OBJETIVOS

3.1 Geral

Estimar a prevalência da infecção pelo VHB e VHD em cinco municípios do estado do Maranhão (Axixá, Morros, Icatu, Humberto de Campos e Urbanos Santos)

3.2 Específicos

Estimar a prevalência da infecção de hepatite delta

Descrever a soroprevalência da hepatite B

Relacionar a prevalência de hepatite B com fatores de risco

4 METODOLOGIA

4.1 Tipo de estudo

Estudo transversal analítico

4.2 Período e local de estudo

A pesquisa foi realizada nos municípios maranhenses de Urbano Santos, Axixá, Morros, Icatu e Humberto de Campos. Fazendo parte ainda dos locais de estudo o Centro de Pesquisa Clínica do Maranhão (CEPECMA), no Laboratório Central de Saúde Pública do Maranhão (LACENMA), no Instituto de Medicina Tropical da de São Paulo da Universidade de São Paulo (IMTSP), no período de março de 2012 a junho de 2016.

4.3 Desenho do estudo e número amostral

Para o cálculo da amostra foi adotada uma prevalência de 0,5% do HBsAg, considerando o resultado da prevalência do HBsAg, encontrada no inquérito nacional na região nordeste (BRASIL, 2011), com erro absoluto de 0,3%, nível de confiança de 95% assumindo um efeito de desenho igual a 2.

A seleção dos participantes da pesquisa ocorreu através de amostragem por conglomerado, no qual os municípios foram divididos em setores censitários baseados nos dados do IBGE. De posse do mapa de cada setor, foi sorteado o quarteirão inicial, em seguida o ponto de início do quarteirão e o lado do percurso. Em cada setor, eram coletadas 134 amostras.

Participaram do estudo 3983 indivíduos residentes nos municípios de Humberto de Campos, Urbano Santos, Axixá, Morros e Icatu, com residência fixa há pelo menos seis meses e que consentiram por escrito com sua participação ou responsável (Termo de Consentimento Livre e Esclarecido), após esclarecimento dos objetivos e metodologia do estudo em foco, com idade mínima de um ano.

4.4 Instrumentos de coleta e avaliação de dados

Os dados foram obtidos através do questionário e exames sorológicos.

4.4.1 Questionários

Foram realizadas as entrevistas por dois pesquisadores, os quais foram treinados para sua aplicação padronizada, mediante um questionário epidemiológico demográfico que incluem variáveis (idade, sexo, ocupação, naturalidade, tempo de residência no município, viagens, cor, religião, estado civil, ocupação, renda familiar,) e epidemiológico/clínico (história de icterícia, fatores de risco associados com a transmissão de vírus hepatotrópicos, história de hepatite) (APÊNDICE A).

4.4.2 Coleta de material biológico

Foram coletadas amostras de 10 mL de sangue com anticoagulante, processados e separados o soro. Posteriormente foram feitas alíquotas em duplicata e conservadas em freezer a -80ºC para realização dos exames sorológicos.

As referidas amostras foram armazenadas no Centro de Pesquisa Clínica do Hospital Universitário da Universidade Federal do Maranhão (CEPEC-HU-UFMA).

4.4.3 Exames sorológicos

Os exames sorológicos foram realizados no Laboratório Central do Estado do Maranhão (LACEN), todas as amostras foram submetidas à realização do HBsAg, anti-HBc e anti-HBs, através do ensaio imunoenzimático (ELISA), utilizando kits comerciais da DIASORIN.

Para a investigação do anti-HDV, foi realizado ELISA das amostras positivas para HBsAg.

4.5 Análise dos dados

O banco de dados foi construído no Microsoft Excel 2010 e foram analisados utilizando o programa SPSS® 21.0 e o Stata® versão 12.0 (Stata Corp., College Station, USA). Foram realizada análise descritiva de portadores de sorologia positiva para VHB e VHD. Uma análise multivariada foi realizada através de Regressão de Poisson, com cálculo de correção para efeito cluster com nível de significância <0,05. Essa análise foi realizada para identificar fatores associados à prevalência do VHB.

As variáveis sócio demográficas foram município (Axixá; Morros; Humberto; Icatu; Urbano), zona (urbana; rural), sexo (feminino; masculino), cor autorreferida (preta; branca; parda; amarela), idade (1 a 5 anos; 6 a 15 anos; 16 a 30 anos; 31 a 60 anos; mais de 60 anos), situação conjugal (solteiro; casado; divorciado; viúvo; união estável), religião (católica; evangélica; outra; nenhuma; ignorado), renda familiar (menos de 1 salário mínimo; de 1 SM a 3 SM; 4 ou mais SM; ignorado), transfusão sanguínea (sim; não; não sabe), consumo de álcool (sim; não), consumo de drogas ilícitas (usa; já usou; nunca usou; ignorado), compartilhamento de perfuro cortantes (sim; não), tatuagem (tem; não tem), piercing (tem; não tem), acumpuntura (sim; não), vida sexual (nunca teve; ativa; inativa), uso de preservativo (sempre; às vezes; nunca; ignorado; não se aplica), número de parceiros (apenas 1; mais de 1; ignorado; não se aplica), mais de 3 parceiros nos últimos 6 meses (sim; não; ignorado; não se aplica).

As sorologias foram categorizadas em positivo e negativo, utilizando como variável dependente o marcador de contato prévio com o VHB, o anti-HBc.

4.6 Considerações éticas

Este estudo foi submetido à Comissão Científica do HUUFMA, à análise do Comitê de Ética em Pesquisa do HUUFMA e tem Parecer Consubstanciado aprovado sob o nº 448.731 (ANEXO A) após emenda (via Plataforma Brasil) que solicitou prorrogação do tempo de execução do projeto, atendendo aos requisitos da Resolução 466/12 do Conselho Nacional de Saúde/Ministério da Saúde.

Somente após a autorização de cada participante, com a assinatura do Termo de Consentimento Livre e Esclarecido (Apêndice B), foi realizada a coleta

das informações por meio de um questionário, assim como a coleta de sangue para a realização dos exames laboratoriais. Os procedimentos de biossegurança referentes à coleta, manipulação e processamento do material biológico foram realizados segundo as regras básicas para o trabalho em laboratório (BRASIL, 2006).

4.7 Recursos Financeiros

A fonte de recursos assegurada para o desenvolvimento deste projeto provém dos editais da Fundação de Amparo à Pesquisa do Estado do Maranhão (FAPEMA) PPSUS-03348/13 e PPP-01263/12; Departamento de DST, AIDS e Hepatites Virais-Secretária de Vigilância em Saúde/Ministério da Saúde/Escritório das Nações Unidas Sobre Drogas e Crime. A Secretaria do Estado da Saúde do Maranhão (SES).

5. RESULTADOS

5.1 CAPÍTULO I – Artigo 1- Unexpected findings of Hepatitis B and Delta infection in Northeastern Brazil: a public health alert

5.1.1 Nome do Periódico com sua classificação na WEBQUALIS da CAPES (A1, A2, B1 ou B2) na área de Avaliação Medicina I- Journal of Viral Hepatitis (A1)

Journal of Viral Hepatitis

Unexpected findings of Hepatitis B and Delta infection in Northeastern Brazil: a public health alert.

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Keywords:	Hepatitis B, Hepatitis Delta, Associated factors, Northeastern Brazil, Maranhão

Unexpected findings of Hepatitis B and Delta infection in Northeastern Brazil: a public health alert.

Hepatitis B and Delta in Northeastern Brazil

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ABSTRACT

Aim: To estimate the prevalence rates of hepatitis B (HBV) and Delta viral (HDV), infection in five municipalities of Maranhão, Northeastern Brazil, and to identify the associated factors. **Methods:** A total sample of 3856 to 4000 individuals participated of this study. Questionnaires were administered to identify sociodemographic characteristics and factors associated with transmission. Patient sera were tested for the markers HBsAg, anti-hepatitis B core antigen (anti-HBc), anti-HBs and anti-HDV (only in HBsAg-positive sera) by enzyme-linked immunosorbent assay (ELISA). Factors associated with HBV were detected by means of multivariate Poisson regression. **Results:** Overall, 3983 subjects were included. Ninety-two of the participants were HBsAg-positive (2.30 % 95 % CI: 1.80-2.80), and anti-HBc was detected in 1535 subjects (38.50 % 95 % CI: 37-40). The factors associated with the presence of anti-HBc were the following: (1) Municipality ($P < 0.001$); Age ($P < 0.001$); School education ($P < 0.001$); Illicit drug use ($P = 0.001$); HBV vaccine ($P = 0.041$). Among the HBsAg carriers, eight were anti-HDV-positive (8.69 %; 95 % CI = 2.90-14.40). **Conclusion:** HBV exhibited intermediate endemicity in the studied region. Traditional factors were associated with exposure to the virus. The presence of the Delta virus was confirmed.

Keywords: Hepatitis B, Hepatitis Delta, Associated Factors, Northeastern Brazil, Maranhão

1 INTRODUCTION

In 2015, hepatitis caused by the hepatitis B virus (HBV) caused 887,000 deaths, especially due to complications. This occurrence is because 257 million people in the world have a chronic infection [1]. HBV is a DNA virus that is transmitted mainly by contact with contaminated blood and genital secretions through the parenteral, perinatal, sexual and horizontal routes [2,3].

According to the WHO, the endemicity of HBV infection is determined by hepatitis B surface antigen (HBsAg) seroprevalence, which is classified as high (more than 7 % of the population), medium or intermediate (2 % to 7 %) and low (below 2 %) [1]. The above-described endemicity depends on the person's age at the time of infection and the predominant transmission route in that population [4].

Although pregnant women are routinely submitted to HBV screening and despite effective vaccination against HBV since the early 1980s, the availability of these services is distributed in a very heterogeneous manner worldwide. Thus, regions with endemicity as high as 8.83 % in Africa and 5.26 % in the Western Pacific region still exist [5].

In Brazil, a recent national survey (2004-2009) in all state capitals found HBsAg seroprevalence rates of 0.63 %, 0.48 %, 0.37 %, 0.31 %, 0.31 % and 0.26 % in the North, South, Northeast, Central-West and Southeast regions and in the Federal District, respectively [6,7]. The results of the above study were included in the review of Ott *et al*. (2012) and Schweitzer *et al*. (2015) and were pivotal for defining Brazil as a country with low endemicity [8].

A highly complicating factor in chronic HBV infection is co-infection with the hepatitis Delta virus (HDV), which causes a worse evolution of hepatic disease [9,10,11].

HDV is an RNA virus, and its main transmission route is parenteral. It requires HBsAg for infectivity and survival [12,13]. Its global distribution is also heterogeneous; however, despite its link to HBV, their endemicity rates do not always coincide [14]. HBV control by vaccination has also aided in reducing HDV-borne disease in developed countries but HDV infection is still highly relevant in some developing regions worldwide, such as the Brazilian Amazon and many African countries, where the prevalence of anti-hepatitis Delta antigen among HBsAg-positive individuals can reach 40 % [14,15].

Until recently in Brazil, HDV was thought to be present only in the Western Amazon. However, after studying a sample of HBV-positive individuals in Maranhão State, five cases

of HBV-HDV co-infection were detected among patients from a specific region of the state, and surprisingly, the HDV-8 genotype was identified, which had never been described among individuals born outside Africa [16]. In the above population, the HBV genotype D4 was also found in co-infections with HDV-8, suggesting that slave trade might have been responsible for the introduction of these viruses in Maranhão State [17]. Thus, the present study conducted a population-based survey in five municipalities of the above-mentioned region, aiming to estimate the prevalence rates of HBV and HDV infection and to determine the factors associated.

2 MATERIAL AND METHODS

2.1. Study population.

The present work is a population-based prevalence study conducted in the Northeast region of Brazil, in the municipalities Urbano Santos (24,573 inhabitants), Axixá (11,407 inhabitants), Morros (17,783 inhabitants), Icatu (25,145 inhabitants) and Humberto de Campos (26,189 inhabitants) of Maranhão State, in the period of March 2012 to June 2016.

Subjects with at least one year of age and living for at least six months in the studied municipalities were included. The sample was calculated using a 0.5 % prevalence of HBsAg (considering the result of the national survey on HBsAg prevalence in the Northeast region [6]), a 0.3 % absolute error, a 95 % confidence interval (95 % CI) and a design effect of 2, thus totaling a sample of 3,856 to 4,000 individuals.

Participants were selected via cluster sampling, for which municipalities were divided into sectors. With a map of each sector, the first block was drawn, followed by the starting point of the block and then the route. If a block did not render enough samples for the sector, an additional block was drawn, and this cycle was repeated until the sample size of each sector was achieved.

2.2. Data collection.

Individual data on socioeconomic and demographic variables, risk factors, alcohol use and hepatitis B vaccination status (assessed by viewing the participant's vaccination card)

were collected by trained interviewers who administered a structured questionnaire during home visits.

2.3. **Laboratory tests.**

Blood samples collected after the interview were submitted to enzyme-linked immunosorbent assay (ELISA) with commercial kits (Diasorin®, Italy) to detect HBsAg, anti-hepatitis B core antigen (anti-HBc), anti-HBs and anti-HDV in HBsAg-positive samples.

2.4. **Statistical analysis.**

In the data analysis, anti-HBc was the dependent variable. The relative risk was estimated by means of a multivariate Poisson regression with a robust variance fit, and 95 % CIs were calculated.

Variables were selected for the model if they exhibited $P < 0.2$ in the non-fitted analysis. There were no criteria for the removal of variables. Since more than one person per home could be interviewed, estimates were corrected for clustering or agglomeration. Data analysis was performed with Stata® software, version 12.0 (Stata Corp., College Station, TX, USA). The level of significance was set at 0.05.

2.5. Ethics committee.

This project was approved by the Human Research Ethics Committee of the University Hospital of the Federal University of Maranhão under the number 448.731.

3 RESULTS

Overall, 3983 subjects were included in the study: 1496 (37.6 %) were living in urban and 2487 (62.4 %) in rural areas. Table 1 shows the frequency distributions of demographic (municipalities), socioeconomic (sex, skin color, age, school education, marital status and family income) and epidemiological factors (history of blood transfusion, alcohol use, drug use, needle sharing, tattoos, piercings, acupuncture, sex life, condom use, number of partners and number of hepatitis B vaccine doses) in the studied population.

Prevalence: HBsAg, anti-HBc and anti-HBs prevalence rates are described in Table 2. Among the 92 HBsAg carriers, three (3.26 %) exhibited atypical serological profiles (coexistence of HBsAg and anti-HBs) and have been described in a previous paper [18]. The overall prevalence of anti-HBc was 38.5 % (95 % CI: 37.0–40.0). The studied municipalities exhibited differences in prevalence: Axixá = 17.5 % (95 % CI: 14.4 – 20.9), Morros = 44.8 % (95 % CI: 41.4 – 48.3), Icatu = 40.3 % (95 % CI 36.6 – 44.1), Humberto de Campos = 43.1 % (95 % CI: 40.2 – 46.1) and Urbano Santos = 38.8 % (95 % CI: 35.1 – 42.6).

Among HBsAg carriers, eight were positive for anti-HDV (8.69 %; 95 % CI = 2.90-14.40), among which four were from the municipality of Humberto de Campos, two from Morros and two from Urbano Santos.

Table 3 exhibits the results of non-fitted analysis between the studied variables and positive anti-HBc, with the respective incidence-rate ratios (IRRs) and 95 % CIs. The reference category was the one with the lowest transmission risk, and variables with $P < 0.2$ were selected for the fitted model.

Table 4 shows the results of multivariate analysis. Compared to Axixá, the municipality of Morros exhibited a two-fold higher risk of contact for anti-HBc (IRR = 2.54, 95 % CI= 2.02-3.20), followed by Humberto de Campos (IRR = 2.1, 95 % CI = 1.52-2.90), Urbano Santos (IRR = 1.66, 95 % CI= 1.16-2.36) and Icatu (IRR = 16, 95 % CI = 1.05-2.43). Age was the associated factor, and an age above 60 years increased the risk three-fold (IRR = 3.03, 95 % CI =2.13-4.29). School education was also associated, and five or more years of school education exhibited higher protection against infection (IRR = 0.74, 95 % CI = 0.65-0.84). Illicit drug use (IRR = 1.36, 95 % CI = 1.15-1.60) and incomplete vaccination were also associated with contact with HBV.

4 DISCUSSION

The present study, which involved five municipalities of Maranhão State (Northeastern Brazil) and included individuals aged one year and above, identified a 2.3 % (95 % CI 1.8–2.7) seroprevalence of HBsAg, and eight (8.69 %; 95 % CI 2.90 -14.40) of the

HBV carriers were also seropositive for anti-HDV. These results confirmed the notions that the endemicity of HBV in the studied region was higher than expected for the Northeast region of Brazil (0.37 %) and that there was, in fact, evidence of a significant presence of HDV.

The seroprevalence of HBsAg found in the present work is different from the current concept that Brazil has low endemicity for HBV (0.65 %), according to the last systematic review of papers published between 1965 and 2013 [5], but confirms the information that Brazil still exhibits regions with endemicity above 2 %, as reported by Souto (2016) in a recent systematic review of 100 Brazilian studies [19]. This prevalence applies especially to rural areas with precarious socioeconomic conditions, as is the case of the studied region of the present work. Specifically, in Maranhão State, a survey in a quilombola community (descendants of African slaves) found an even higher seroprevalence of HBsAg (12.5 %) [20]. These variations in Brazil clearly reflect the differences in economic indices, demography, cultural factors and access to health-care services in the country, which have already been described in regions with similar socioeconomic conditions [3].

In the evaluation of anti-HBc seropositivity (an indicator of the overall HBV infection rate), the sample exhibited a value of 38.5 %, which is also above the means of both the nation and the Northeastern region, which are estimated at 11.6 % and 11.7 %, respectively [6,19], but is in agreement with the findings of a study from another municipality of the rural zone of Maranhão State (40.7 %) [21], which is a different region from that studied in the present study. This finding suggests that Maranhão State is, in fact, a Brazilian state where HBV infection is a more significant aggravation than what is considered for most regions of the country because the results found here are equal to or above those of recent Brazilian studies on high-risk populations, including prison inmates [22,23], HIV-positive individuals or those with coagulopathies that require frequent blood transfusions [24].

Among the factors associated with higher HBV infection rates were the municipality of residence, older age, lower education level, history of illicit drug use, and absence of vaccines or incomplete vaccinations.

Living in the municipalities of Icatu, Humberto de Campos, Morros or Urbano Santos was independently associated with a higher risk of HBV infection compared with the municipality of Axixá. Even though these municipalities are contiguous, Axixá is classified as having a medium municipal human development index (HDI), whereas the remaining studied

municipalities have low municipal HDIs (Brazilian Institute of Geography and Statistics; Instituto Brasileiro de Geografia e Estatística – IBGE). Lower socioeconomic indices have been associated with higher prevalence of HBV infection, especially when associated with the possibility of horizontal transmission due to inadequate habits of hygiene and disease prevention [25,26], which could explain the results found in the studied region of the present work.

In the same line of reasoning as the above, a higher education level, an important indirect indicator of socioeconomic development, was independently associated with a lower risk of HBV infection. These results have been observed in other national surveys [7,27,28] and even in developed countries, such as Italy [29], which further supports the concept that education level is a protective factor for specific infecto-contagious diseases because it is associated with better understanding of risky sexual behaviors, improving the prevention of sexually transmitted diseases (STDs) [30]. It is important to note that a lower education level is directly associated with lower awareness of harboring chronic HBV infection [31] and with a lower vaccination rate [32], thus perpetuating horizontal and vertical virus transmission.

Older age, both here and in other regions of the world [6,7,29], has been associated with HBV infection, thus representing higher chances of exposure to the virus, with sexual activity and with the fact that older individuals have not been submitted to vaccines, which, in Brazil, became compulsory in 1998.

Among the classical risk factors of infection, such as sexual behavior, use of sharp non-disposable materials, and history of blood transfusions [33], only illicit drug use was clearly associated with anti-HBc in this population. Another interesting finding is that most of the individuals who reported the above habit used marijuana or cocaine rather than injection drugs. Thus, this behavior might be associated with mechanisms of transmission that could not be clearly identified here, suggesting that these individuals must be prioritized for prevention.

In the present work, with participants aged one year and above (many with a complete vaccination schedule), we chose to include data on vaccination (yes or no) and the number of registered doses due to the lack of information in the country regarding the effect of the vaccine on the prevention of hepatitis B in the general population. The universal vaccination initiated during the first year of life in Brazil as of 1998 already included populations at risk and was progressively extended until, in 2015, all residents of the country

were being covered [34]. As expected, the higher the number of vaccine doses the individual was submitted to, the lower the frequency of anti-HBc was. However, among those aged 30 years and above (1539 individuals), only 15 % exhibited positivity for anti-HBs alone during data collection, thus demonstrating low vaccination coverage in this age range (a paper addressing the evaluation of the results of vaccination is being elaborated).

One of the most important results of the present study was the confirmation that HDV infection is a reality in Maranhão State since eight of the 92 HBsAg carriers were positive for anti-HDV. Previously published studies describe the HDV and HBV phenotypes identified among these 92 HBV carriers, which were confirmed as HDV-8 and HBV-D4 [35,36], as had been suspected. Since there were few HDV cases, the factors associated with infection could not be identified. However, cases were identified in the municipalities of Morros, Humberto de Campos and Urbano Santos. The prevalence of 8.6 % in the present study was slightly above that found in Western Europe [37] but was not as high as that described in some African and Asian countries [15,38] and even in the Brazilian Amazon [39]. However, this is one of the few studies with a large sample of the general population of the country outside the Amazon and shows that HDV is a complicating factor that is associated with all of the others identified in Brazil, thus justifying the efforts in which the country is engaged, following the strategy of the WHO to combat viral hepatitis, which is a major global health problem [1], as emphasized in the last World Hepatitis Summit [40].

Of note, the present work has a very representative sample of the population and shows intermediate endemicity of HBV related with living conditions of the population and with the lack of robust preventive measures.

The present work has limitations and strengths. The main strength is the representative sample of these municipalities, given that the main sample was calculated using a prevalence of 0.5 % for HBsAg, which is almost one-fifth of the real prevalence, thus allowing a safe analysis of the prevalence and associated factors. Among the limitations are those related to cross-sectional studies and a possible bias of information on sexual behavior and drug use because information was collected via interviews during a single home visit. Precautions, such as training the interviewers and performing individual interviews, were taken in an attempt to reduce these limitations, although they might not have been sufficient to avoid distortions.

The findings of the present survey reinforce the heterogeneity of prevalence outside the Brazilian capitals and suggest factors that are associated with HBV transmission, showing the need to improve the use of high-impact strategies already regulated in the country, such as the immunization of children, adolescents and adults, in addition to strategies for the screening of infected individuals and for the prevention of vertical transmission, considering the implementation of new policies for populations at risk, such as illicit drug users. Our results also reinforce the need for further research to determine the real prevalence of HDV infection in the country.

Conflict of interests

The authors declare no conflicts of interest (financial, personal, scientific, assistance, educational, religious and social) that could interfere with the results of the survey.

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Table 1. Profile of demographic, socioeconomic and epidemiological variables of hepatitis B. Maranhão State, Brazil, 2012-2016 (*n* = 3983).

Variables	*n*	%
Municipality		
Axixá	532	13.4
Morros	802	20.1
Humberto de Campos	1064	26.7
Icatu	654	16.4
Urbano Santos	931	23.4
Sex		
Male	1680	42.2
Female	2303	57.8
Skin Color		
White	474	11.9
Black	651	16.3
Brown	2858	71.8
Age (years)		
1 to 15	1381	34.7
16 to 30	1063	26.7
31 to 60	1195	30.0
60 and above	344	8.6
Education Level		
Illiterate	326	8.3
1 to 4 years	2391	60.8
5 years or more	963	24.5
Does not apply	253	6.4
Marital Status		
No partner	2455	62.4
Married	744	18.9
Unmarried union	738	18.7
Family Income (minimum wage)		
More than 1	1712	43.0
Less than 1	2119	53.2
Ignored	152	3.8
Blood Transfusion		
No	3762	95.4
Yes	180	4.6

Alcohol Use		
No	2762	69.3
Yes	1221	30.7
Illicit Drug Use		
Has never used	2378	59.7
Has used	299	7.5
		Continues
Uses	332	8.3
Ignored	974	24.5
Shared Needles		
No	1596	40.1
Yes	2387	59.9
Tattoos		
No	3852	96.7
Yes	131	3.3
Piercings		
No	3952	99.2
Yes	31	0.8
Acupuncture		
No	3974	99.8
Yes	9	0.2
Sex Life		
Has never had sex	1607	40.3
Active	1887	47.4
Inactive	489	12.3
Condom Use		
Always	459	11.5
Sometimes	743	18.7
Never	933	23.4
Ignored	241	6.0
Does not apply	1607	40.3
Number of Partners		
Only 1	1752	44
More than 1	243	6.1
Ignored	381	9.6
Does not apply	1607	40.3
Promiscuity (more than two partners in six months)		
No	1638	41.1
Yes	209	5.2
Ignored	170	4.3
Does not apply	1966	49.4
Number of Hepatitis B Vaccine Doses		
3 doses	1047	26.3

2 doses	147	3.7
1 dose	117	2.9
0 doses	1338	33.6
Does not know	1334	33.5

Table 2. Profile of HBV hepatitis serum markers. Maranhão State, Brazil, 2012-2016 (*n* = 3983).

HBV Serology	***n***	**%**	**CI (95 %)**
HBsAg(+) anti-HBc (+)	92	2.3	1.8 – 2.7
anti-HBc(+) anti-HBs(+)	882	22.1	20.8 – 23.4
anti-HBc (+) alone	561	14.1	13.0 – 15.2
anti-HBs (+) alone	800	20.0	18.8 – 21.3
Negative markers	1648	41.5	39.8 – 42.9
Total	3983	100	

Table 3: Non-fitted analysis of factors associated with hepatitis B viral infection (anti-HBc-positive). Maranhão State, Brazil, 2012-2016 (*n* = 3983).

Variables	*N*	IRR	95 % CI	*P* value
Municipality				< 0.001
Axixá	532	1	-	
Morros	802	2.56	1.90 - 3.46	
Humberto	1064	2.47	1.61 - 3.79	
Icatu	654	2.30	1.47 - 3.62	
Urbano	931	2.18	1.45 - 3.28	
Sex				0.319
Male	1680	1	-	
Female	2303	0.94	0.87 - 1.02	
Skin Color				0.008
White	474	1	-	
Black	651	0.86	0.71 - 1.04	
Brown	2858	0.83	0.73 - 0.94	
Age				< 0.001
1 to 15 years	1381	1	-	
16 to 30 years	1063	2.97	2.31 - 3.82	
31 to 60 years	1195	5.22	3.66 - 7.45	
60 years and above	344	6.40	4.35 - 9.41	
Education Level				< 0.001
Illiterate	326	1	-	
1 to 4 years	2391	0.52	0.46 - 0.59	
5 years or more	963	0.47	0.40 - 0.56	
Does not apply	253	0.09	0.04 - 0.18	
Marital Status				< 0.001
No partner	2455	1	-	
Married	744	2.48	2.16 - 2.86	
Unmarried Union	738	2.23	2.01 - 2.48	
Family Income (minimum wage)				0.818
More than 1	1712	1	-	
Less than 1	2119	1.04	0.90 - 1.21	
Ignored	152	0.94	0.68 - 1.30	
Blood Transfusion				0.016
No	3762	1	-	
Yes	180	1.31	1.05 - 1.64	
Alcohol Use				< 0.001
No	2762	1	-	
Yes	1221	1.70	1.57 - 1.84	
Illicit Drug Use				< 0.001

Continues

Has never used	2378	1	-	

Continued

Has used	299	1.23	1.01 - 1.50	
Uses	332	1.51	1.25 - 1.83	
Ignored	974	1.30	1.06 - 1.58	
Sharing Needles				0.004
No	1596	1	-	
Yes	2387	1.19	1.05 - 1.35	
Tattoos				0.512
No	3852	1	-	
Yes	131	0.88	0.62 - 1.26	
Piercing				0.207
No	3952	1	-	
Yes	31	0.58	0.25 - 1.34	
Acupuncture				0.778
No	3974	1	-	
Yes	9	0.86	0.31 - 2.36	
Sex Life				< 0.001
Has never had sex	1607	1	-	
Active	1887	3.83	3.11 - 4.71	
Inactive	489	3.91	3.04 - 5.04	
Condom Use				< 0.001
Always	459	1	-	
Sometimes	743	1.19	1.02 - 1.39	
Never	933	1.52	1.32 - 1.76	
Ignored	241	1.41	1.22 - 1.64	
Does not apply	1607	0.34	0.27 - 0.41	
Number of Partners				< 0.001
Only 1	1752	1	-	
More than 1	243	0.93	0.86 - 1.01	
Ignored	381	1.01	0.91 - 1.10	
Does not apply	1607	0.26	0.21 - 0.32	
Promiscuity (more than two partners in six months)				< 0.001
No	1638	1	-	
Yes	209	0.96	0.83 - 1.11	
Ignored	170	0.86	0.66 - 1.12	
Does not apply	1966	0.40	0.34 - 0.46	
Number of Hepatitis B Vaccine Doses				< 0.001
3 doses	1047	1	-	
2 doses	147	1.99	1.46 - 2.69	
1 dose	117	2.69	1.95 - 3.70	
0 doses	1338	2.76	1.99 - 3.83	
Does not know	1334	2.45	1.80 - 3.32	

Table 4: Factors associated with hepatitis B viral infection (anti-HBc-positive), multivariate analysis. Maranhão State, Brazil, 2012-2016 (*n* = 3983).

Variables	*N*	IRR	95 % CI	*P* value
Municipality				**< 0.001**
Axixá	532	1	-	
Morros	802	2.54	2.02 - 3.20	
Humberto	1064	2.1	1.52 - 2.90	
Icatu	654	1.60	1.05 - 2.43	
Urbano	931	1.66	1.16 - 2.36	
Sex				0.478
Male	1680	1	-	
Female	2303	0.96	0.88 - 1.05	
Skin Color				0.062
White	474	1	-	
Black	651	0.90	0.77 - 1.06	
Other	2858	0.89	0.81 - 0.99	
Age				**< 0.001**
1 to 15 years	1381	1	-	
16 to 30 years	1063	2.04	1.56 - 2.68	
31 to 60 years	1195	3.03	2.13 - 4.29	
60 years and above	344	3.46	2.45 - 4.88	
Education Level				**< 0.001**
Illiterate	326	1	-	
1 to 4 years	2391	0.94	0.86 - 1.04	
5 years or more	963	0.74	0.65 - 0.84	
Does not apply	253	0.53	0.29 - 0.96	
Marital Status				0.383
No partner	2455	1	-	
Married	744	1.06	0.96 - 1.17	
Unmarried union	738	1.07	0.97 - 1.17	
Blood Transfusion				0.970
No	3762	1	-	
Yes	180	1.01	0.87 - 1.14	
Alcohol Use				0.310
No	2762	1	-	
Yes	1221	1.05	0.95 - 1.16	
Illicit Drug Use				**0.001**
Has never used	2378	1	-	
Has used	299	1.24	1.05 - 1.45	
Uses	332	1.41	1.16 - 1.71	

Continues

Continued

Ignored	974	1.36	1.15 - 1.60	
Sharing Needles				0.979
No	1596	1	-	
Yes	2387	1.01	0.91 - 1.10	
Sex Life				0.372
Has never had sex	1607	1	-	
Active	1887	1.37	0.87 - 2.15	
Inactive	489	1.29	0.84 - 2.01	
Condom Use				0.150
Always	459	1	-	
Sometimes	743	1.04	0.91 - 1.20	
Never	933	1.13	0.99 - 1.30	
Ignored	241	1.12	0.90 - 1.39	
Does not apply	1607	*	*	
Number of partners				0.767
Only 1	1752	1	-	
More than 1	243	1.04	0.94 - 1.14	
Ignored	381	1.06	0.89 - 1.25	
Does not apply	1607	1.10	0.75 - 1.61	
Promiscuity (more than two partners in six months)				0.613
No	1638	1	-	
Yes	209	1.01	0.89 - 1.15	
Ignored	170	0.94	0.80 - 1.12	
Does not apply	1966	0.94	0.82 - 1.07	
Number of Hepatitis B Vaccine Doses				**0.041**
3 doses	1047	1	-	
2 doses	147	1.31	1.03 - 1.67	
1 dose	117	1.39	1.07 - 1.82	
0 doses	1338	1.19	1.03 - 1.37	
Does not know	1334	1.23	1.05 - 1.44	

5.2 CAPÍTULO II – Artigo 2- The hepatitis delta genotype 8 in Northeast Brazil: The North Atlanticslave trade as the potential route for infection

5.2.1 Nome do Periódico com sua classificação na WEBQUALIS da CAPES (A1, A2, B1 ou B2) na área de Avaliação Medicina I- Virus Research (B1)

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The hepatitis delta genotype 8 in Northeast Brazil: The North Atlantic slave trade as the potential route for infection

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ABSTRACT

Hepatitis Delta virus (HDV) is not well known, even though HDV and Hepatitis B virus (HBV) co-infection leads to severe forms of acute and chronic liver diseases. HDV is endemic in the Western Amazon region. Recently, the HDV genotype 8 was found in chronic patients followed at the center for liver studies in the Northeast Brazil, Maranhão. Previous studies suggested that this genotype was introduced in Maranhão during the slave trade. The presence of HDV in that study, which was done outside the Amazon region, led us to investigate whether the virus is found infecting individuals in other regions of Maranhão as well. Thus, we screened ninety-two HBsAg positive individuals from five Municipalities of Maranhão for anti-HD antibody and eight were found positive (8.7%). These eight positive individuals were submitted to polymerase chain reaction (PCR) to investigate active HDV infection. Half of them were positive for a fragment sequence of the delta antigen; their sequence samples were submitted to genotype characterization by phylogenetic analysis. All sequences clustered in a unique branch of the tree separated from the other branch described in Africa. Our study confirmed the presence of HDV-8 in Maranhão. These infected individuals had no evidence of contact with African people. Furthermore, we found individuals infected with HDV-8 in two more different municipalities. More studies like ours are urgent because the co-infection HBV/HDV is more difficult to treat. Identification of the endemic regions and implementation of healthy policies for preventing this infection are urgent in this region.

1. Introduction

Hepatitis Delta virus (HDV) is not well known like other hepatotrophic viruses such as Hepatitis B (HBV) and C (HCV). HDV antigen and antibody were discovered less than four decades ago when a group of physicians investigated serum and liver biopsy of positive HBsAg patients (Rizzetto et al., 1977). HDV is the smallest virion to infect animal cells. The virus has a circular genome of around 1700 base pairs that includes a ribozyme that plays an import role in HDV replication (Alves et al., 2013; Rizzetto and Alavian, 2013; Taylor, 2014). This virus infects liver cells but differently than other known hepatotrophic viruses, it requires the surface antigen (HBsAg) of the HBV in order to enter liver cells and secrete new virions (Sureau, 2006; Taylor, 2014).

HDV is often found in the Mediterranean, Central Africa and Northern parts of South America (Radjef et al., 2004). In the Amazon region of Brazil, HDV, together with HBV, are a major public health burden (Bensabath et al., 1987; Braga et al., 2012). Furthermore, both HDV and HBV have a huge genomic diversion and these viruses split in groups named genotypes (and subgenotypes, for HBV), that show a characteristic geographic distribution (Deny, 2006; Kramvis, 2014). Currently eight genotypes of HDV were described, HDV-1 to HDV-8, with the exception of HDV-1, all genotypes are found in distinct geographic regions (Deny, 2006; Le Gal et al., 2012; Radjef et al., 2004). HDV-2 and 4 are found in the East Asia (Imazeki et al., 1990; Ivaniushina et al., 2001; Sakugawa et al., 1999); the HDV-3 is mainly described in the Amazon Basin (Crispim

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et al., 2014; Gomes-Gouvea et al., 2008; Gomes-Gouvea et al., 2009) and the HDV-5 to HDV-8 were found mainly in African individuals (Deny, 2006; Francois-Souquiere et al., 2016; Le Gal et al., 2012; Radjef et al., 2004).

Although in Brazil HDV is endemic in the Amazon region, mainly in the Western region, recently some cases were found outside this region, in the Maranhão state, Northeast Brazil (Barros et al., 2011). Until now only HDV genotype 3, in addition of few cases of HDV-1, has been identified in Amazon region (Crispim et al., 2014; Gomes-Gouvea et al., 2008; Gomes-Gouvea et al., 2009; Viana et al., 2005), whereas in Maranhão state the African genotype HDV-8 was found infecting Brazilian individuals, which led the authors to suggest that this genotype was introduced in the region during the slave trade (Barros et al., 2011). The history of North Atlantic slave trade in the state of Maranhão corroborates with the same hypothesis of the introduction of the HDV genotype 8 through the slave trade (Silva, 2008).

The presence of HDV in Maranhão, and outside the Amazon region, led us to investigate whether the virus are spread in other municipalities of Maranhão besides those already studied previously (Barros et al., 2011), in order to certify if these findings were not only isolated cases as already found in some regions of Brazil (Mendes-Correa et al., 2011; Strauss et al., 1987). Thus, herein we describe the second report of genotype 8 of HDV in the state of Maranhão, Brazil.

2. Material and methods

2.1. Samples and ethical approval

Ninety-two individuals who were positive for HBsAg serological marker among 3860 individuals, from five municipalities in Northeastern Maranhão, participated in this study (Fig. 1). The research ethnics committee of University Hospital, Federal University of Maranhão (HUUFMA) approved this study and written informed consent was obtained from all individuals that agreed to participate. We screened the 92 samples for anti-HD using the enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (DiaSorin, Italy).

2.2. HDV RNA and HBV DNA amplification and sequencing

HDV RNA and HBV DNA were extracted using the QIAamp MiniElute kit (Qiagen®, Hilden, Germany). Fragments of 403 base pairs (bp) for HDV (partial delta antigen genomic region) and 1306 bp for HBV (partial DNA polymerase- and surface antigen-encoding genes) were amplified by nested PCR (polymerase chain reaction) according to procedures described elsewhere (Gomes-Gouvea et al., 2015; Gomes-Gouvea et al., 2008). PCR fragments were purified using ChargeSwitch® PCR Clean-Up Kit (Life Technologies, USA). Sequencing procedures are described in a previous study (Gomes-Gouvea et al., 2015; Gomes-Gouvea et al., 2008). We performed carefully all procedures to avoid contamination or false-positive results (Kwok and Higuchi, 1989).

2.3. HDV and HBV genotyping

We used the Phred–Phrap software (Ewing and Green, 1998; Ewing et al., 1998) to evaluate the quality of the electropherogram. We obtained consensus sequences from the alignment of the sense and antisense sequences of each strain using CAP3 software available at the web page Electropherogram quality analysis Phred (http://asparagin.cenargen.embrapa.br/phph/).

All HDV and HBV sequences were aligned and edited using the software BioEdit (v. 7.0.8) and the integrated CLUSTAL W program (Hall, 1999). HDV genotypes were classified by phylogenetic reconstructions using the published reference sequences from the GenBank database (http://www.ncbi.nlm.nih.gov/). Phylogenetic analyses were performed using a Bayesian approach, which was done using the Markov Chain Monte Carlo (MCMC) simulation implemented in BEAST v.1.6.1 (Drummond and Rambaut, 2007). The analysis was performed using relaxed uncorrelated lognormal molecular clock and GTR + G + I as nucleotide substitution model; MCMC chains were run for 10 million states, and sampled every 1000 runs to obtain the convergence of parameters. Maximum clade credibility tree was summarized after excluding 10% of burn-in using TreeAnnotator v.1.6.1 and the tree was visualized in FigTree v1.4.2. (Available at: http://tree.bio.ed.ac.uk/software/figtree).

3. Results

3.1. Demographical characteristics of HDV RNA positive individuals

Among the 92 individuals screened for anti-HD antibody, eight were positive (8.7%). Samples from these eight positive individuals were submitted to PCR. Half of them were positive for a fragment sequence of the delta antigen and submitted to sequencing. Only two of them (50%) had detectable HBV DNA and had the HBV subgenotype determined. Among the four HDV RNA carriers, just one was female. Their ages ranged from 23 to 49 years and they lived in three different municipalities (Table 1).

3.2. Genotype distribution

The four successful sequenced strains were submitted to phylogenetic analysis and showed that all sequences clustered, with high posterior probability, with the African genotype 8 together with other two strains found in Maranhão (Fig. 2). All sequences clustered in a unique branch of the tree apart from all the other described in GenBank. Although there were two infected individuals from the same municipality of Humberto de Campos, these sequences did no relate with each other in the tree. The same was found for the sample from Urbano Santos with the ones from our previous study (JF 298899_MA_Brazil e JF 298898_MA_Brazil) (Barros et al., 2011).

Among the four HDV-8 individuals co-infected with HBV, two had detectable HBV DNA by PCR and were sequenced. Both were classified into subgenotype D4 (Table 1).

The GenBank accession numbers for the four HDV sequences described in this study are KX599369–KX599372.

4. Discussion

In Brazil, HDV is a major public health burden in the Western Amazon region, where co-infection with HBV leads to severe forms of acute and chronic liver diseases (Bensabath et al., 1987; Braga et al., 2012; Viana et al., 2005). Conversely, only some cases of HDV infection have been sparsely found in other regions of Brazil (Mendes-Correa et al., 2011; Strauss et al., 1987). The Northeastern region of Maranhão, according to our knowledge, was the first region outside Amazon where a modest frequency of anti-HD serological marker (3.8%; 5/133) was found among positive HBsAg individuals. Also, the African HDV-8 was only found in this Brazilian region (Barros et al., 2011). Thus, this finding led us to investigate whether this genotype could be found in other municipalities of this state.

The HDV genotype 8 was firstly found infecting individuals in France who were born in West and Central Africa (Le Gal et al., 2006; Makuwa et al., 2008; Makuwa et al., 2009). In the present study, four strains were successfully sequenced and classified into

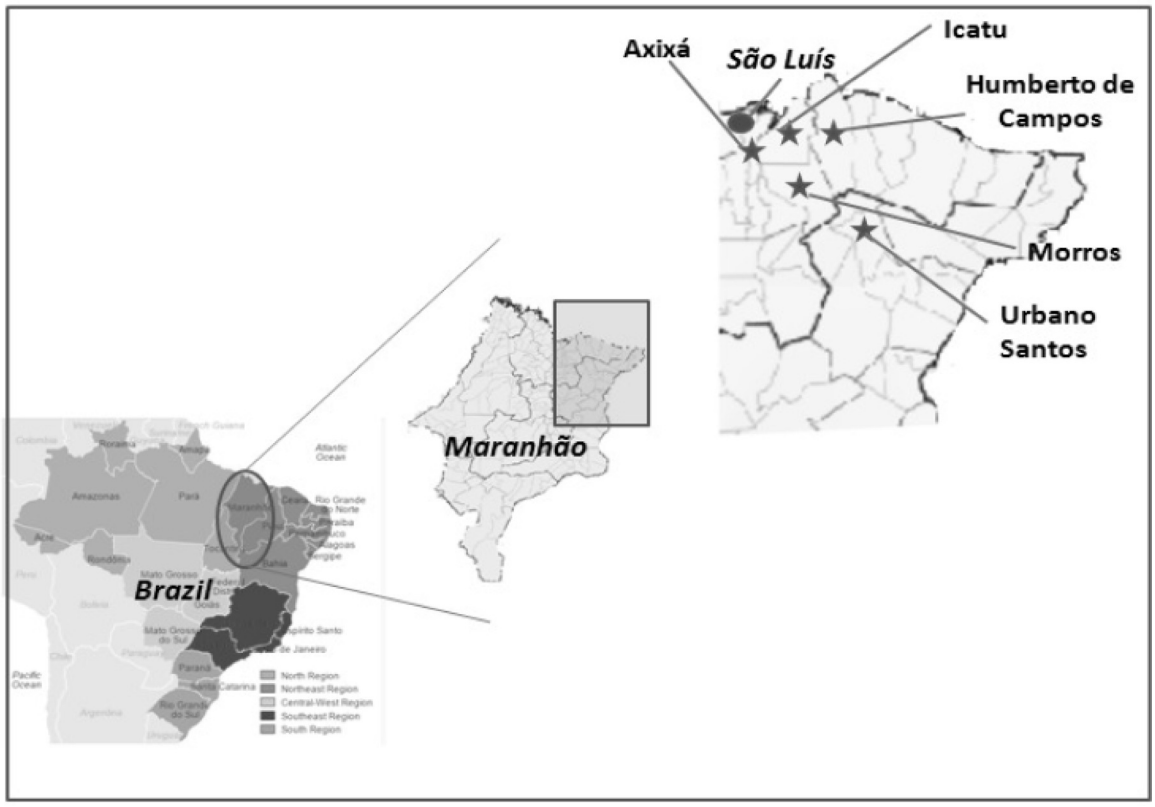

Fig. 1. Geographic localization of the municipalities of this study (stars). The circle indicates the state capital, São Luís.
Source: modified from Wikimedia Commons https://commons.wikimedia.org/wiki/File:Maranhao_MesoMicroMunicip.svg#/media/File:Maranhao_MesoMicroMunicip.svg and https://commons.wikimedia.org/wiki/File:Brazil_Labelled_Map.svg#/media/File:Brazil_Labelled_Map.svg.

Table 1
Demographical characteristic HBV/HDV co-infected individuals described in this study.

ID	Gender	Race*	Age	Origin	Zone	FamilyIncome(MW)	HDV-genotype	HBV-subgenotype
1015	M	Black	23	Morros	Rural	<1 MW	HDV-8	ND
2231	M	Black	25	H. Campos	Rural	<1 MW	HDV-8	D4
2321	M	Black	49	H. Campos	Rural	<1 MW	HDV-8	D4
3959	F	Mestizo/Mullato	39	U. Santos	Urban	<1 MW	HDV-8	ND

*self-declaration; M-Male; F-Female; MW- Minimum Wage; ND-Not determined.

this African genotype 8. Although we have collected samples in the same state, Barros et al. (2011) studied samples that were mostly from the metropolitan region of capital city of Maranhão. This study found only one individual from the capital São Luís who was infected with HDV-3 and also found genotype 8 in two unrelated individuals from the municipality of Urbano Santos, which is a municipality outside the metropolitan region. Similarly to our findings, these infected individuals had no history of travel to other countries; neither they reported to have relatives nor close contact with people from African continent. Furthermore, we found individuals infected with HDV-8 in two additional municipalities located outside the metropolitan region. Thus, it is possible that the virus is endemic not only in this region of Urbano Santos but elsewhere in Maranhão. More studies are required to increase our knowledge on HDV in this region. Identification of endemic regions and implementation of healthy policies to prevent this infection are urgent measures to be taken.

HDV and HBV were discovered with around 10 years apart (Blumberg et al., 1965; Rizzetto et al., 1977). A plausible explanation for so few molecular epidemiology studies on HDV relies on the idea that HBV vaccination that occurred in the 1990s would "send HDV off the match", especially in Europe (Ahan and Gish, 2014; Rizzetto and Ciancio, 2012). Although some studies have been reported regarding the HDV control in Europe, cases of HDV infection are still described in this continent, but mainly among immigrants from endemic areas. In this case, the prevalence of HDV can be increased also by infection among local population of HBV carriers or individuals not vaccinated against HBV (Rizzetto, 2009).

Thus, apart from few HDV cases described outside Amazon region in Brazil, care must be taken in order to avoid HDV introduction and spread in Brazil besides Amazon region. There are few published robust studies that evaluate the prevalence of immunity for HBV (anti-HBs positivity) in Brazil and outside the metropolitan cities of Maranhão. A recent preliminary report published by our group, in two out of tree cities where we found HDV infected individuals, showed that only around 20% (171/871) of the individuals were vaccinated (showed isolated anti-HBs detectable) (Valente et al., 2014). In capital cities of Brazil, studies have shown 86.7% (15,388/17,749) of three-dose HBV vaccination coverage among newborns (Luna et al., 2009) and the utmost of 49.8% of vaccinated people between 20–69 years of age (Ximenes et al., 2015). Thus, it is noteworthy that cities in the hinterland, as these reported in our study, should also be taken into consideration in investigations of vaccination status, as these regions are often less assisted in rela-

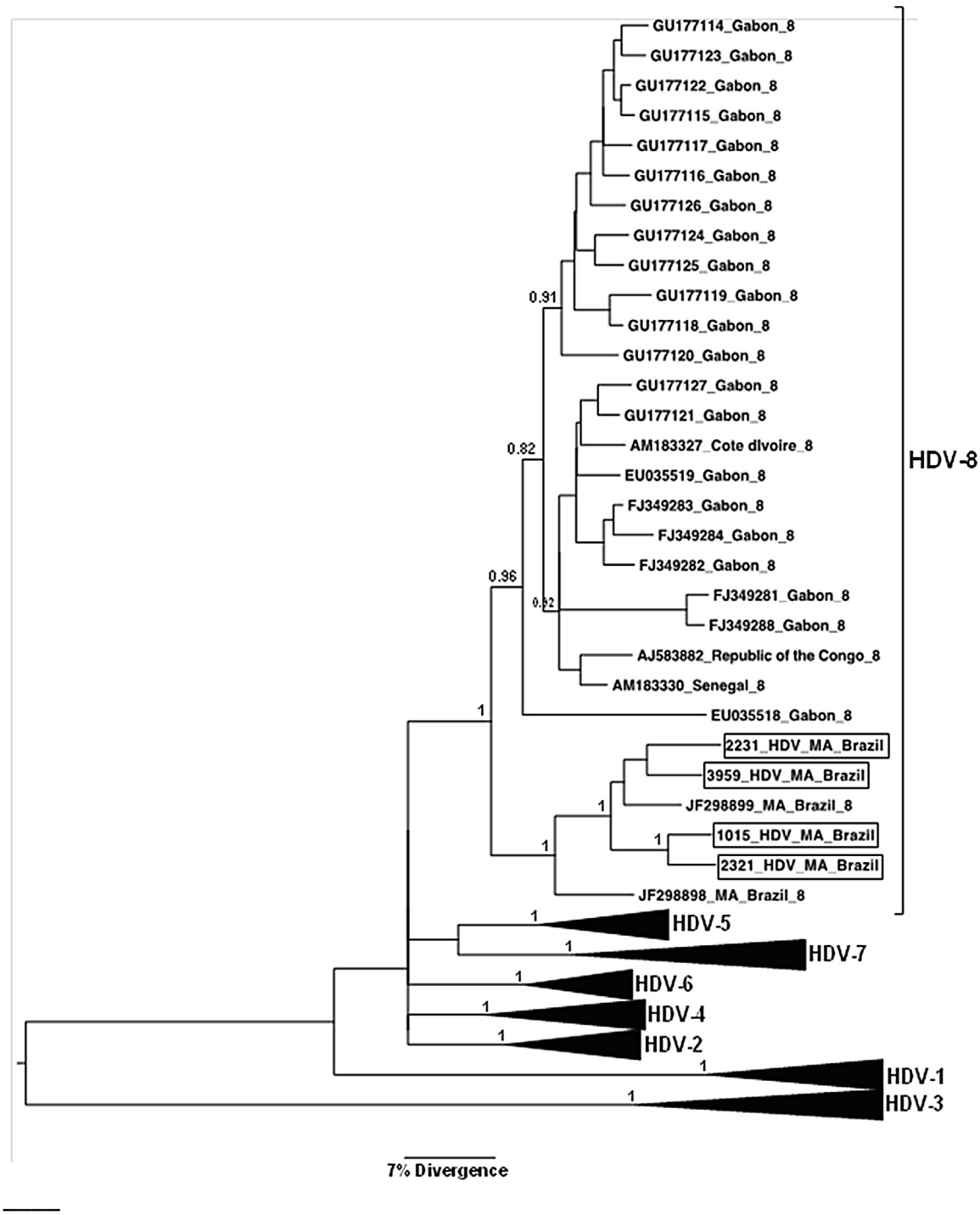

Fig. 2. Phylogenetic analysis of HDV isolates identified in this study (marked by rectangular shape). Sequences retrieved from GenBank are indicated by their corresponding accession number, genotype and geographic origin. The values of posterior probability are shown for key nodes.

tion to health, education and sanitation programs and eventually susceptible to HBV and HDV infections.

In this study we found two individuals with HDV-8/HBV-D4 co-infection. The D4 subgenotype of HBV is rare in the world but more frequently found in Africa and in countries where African slaves took part in the population formation during the trans-Atlantic slave trade period (Andernach et al., 2009; Barros et al., 2011; Brichler et al., 2013; Hubschen et al., 2009; Kwange et al., 2013; Loureiro et al., 2015; Osiowy et al., 2011; Perbi, 2001). Thus, the theory of the origin of these strains in Africa is reinforced with our finding (Barros et al., 2011) although the exact African region of origin of these viruses needs to be still investigated. Our four HDV-8 sequences clustered, with 100% posterior probability, with a unique branch of the tree harboring only the Brazilian strains, apart from all the others from Senegal, Ivory Coast, Republic of the Congo and Gabon. The tree conformation found in the branch of our sequences is different from that found in a recent study of Francois-Souquiere et al. (2016) in Gabon, where phylogenetic analysis shows that samples grouped in two divergent subgroups. Other issue that drew our attention is the fact that the monophyletic pattern found in

HDV-8 was also identified in individuals infected with HBV-D4 in mono or co-infection with HDV-8 (Barros et al., 2014; Barros et al., 2011). Such characteristic suggests that this strain was introduced just once in this region along with HBV subgenotype D4. Our main hypothesis is that our HDV-8 sequences are from a still not known source of infection in Africa. Thus, studies regarding HDV infection are necessary in Africa and in other countries with contemporary contribution of African ethnicity in their population formation.

Because only few studies are available that can contribute to the discussion about the origin of HDV-8 in Brazil (Barros et al., 2011; Francois-Souquiere et al., 2016; Le Gal et al., 2006; Makuwa et al., 2008; Makuwa et al., 2009), the history in the region may explain the link between Brazil and Africa in regard to HDV. Thus, the history of Maranhão, Africa and Caribbean countries, regarding the trans-Atlantic slave trade, may explain the observations regarding these genotypes. In summary, the main cargos of enslaved Africans in Maranhão embarked in the West Africa (Meireles, 2009; Silva, 2008) where the first HDV strains described are from (Le Gal et al., 2006). Some geographic features and navigation issues (wind and ocean currents) made Maranhão adopt the slave trading of North Atlantic countries (Silva, 2008). However, the exact origin of these strains are still an issue of speculation because there are not many HDV-8 sequences available in other regions of Africa rather than Central region (Francois-Souquiere et al., 2016; Makuwa et al., 2008). A recent study of Andernach et al. (2014) in Sub-Saharan African individuals, which included 743 individuals from Burkina Faso, the Central Africa Republic and Chad, investigated HDV infection. Of the twenty-four PCR positive samples sequenced by them, no HDV-8 was described, with predominance of HDV-1 and few sequences of HDV-5 and 6. Thus, only with more studies in different regions of Africa we may ensure the origin of our HDV-8 sequences.

The population of Brazil was formed mainly by three main ancestral backgrounds: Europeans, Amerindians, and Africans, being this last one the main representative in the Northeast Brazil, where Maranhão state is located (Pena et al., 2011). None of the individuals harboring HDV genotype 8 reported to have lived abroad or had close contact with individuals from other countries. Furthermore, all HDV RNA infected individuals in our study declared themselves as black or mullato. Thus, the identification of this genotype in Maranhão seems indeed to be related to the slavery trade (Barros et al., 2011).

Our findings indicate that HDV may be spreading to regions of Brazil other than Amazon. More studies must be done in order to identify the main strains spreading outside Amazon as well to establish the origin of these Brazilian sequences of HDV-8.

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5.3 CAPÍTULO III – Artigo 3- Mutation in the S Gene a Determinant of the Hepatitis B Virus Associated With Concomitant HBsAg and Anti-HBs in a Population in Northeastern Brazil

5.3.1 Nome do Periódico com sua classificação na WEBQUALIS da CAPES (A1, A2, B1 ou B2) na área de Avaliação Medicina I- Journal of Medical Virology (B2)

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Mutation in the S Gene *a* Determinant of the Hepatitis B Virus Associated With Concomitant HBsAg and Anti-HBs in a Population in Northeastern Brazil

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Mutations in the a determinant of S gene may develop co-existence of hepatitis B surface antigen (HBsAg) and antibodies to HBsAg (anti-HBs) in the serum of infected hepatitis B virus (HBV) individuals. Mutations in this region may change the antigenicity of HBsAg, which in turn, lead to escape of neutralizing action of anti-HBs antibodies. This study identified individuals with concomitant HBsAg and anti-HBs serological markers in individuals of Maranhão, Northeastern Brazil. Samples from a population-based study were evaluated for HBsAg, anti-HBs, and anti-HBc, and those that tested positive for simultaneous HBsAg and anti-HBs were submitted to HBV DNA quantification and S gene characterization by Sanger sequencing. Mutations were investigated in the a determinant located in major hydrophilic region (MHR) of the S gene. Among 3,984 samples analyzed, 92 (2.3%) were positive for HBsAg and three had the atypical HBsAg and anti-HBs-positive profile (3.26%). The frequency of HBsAg and anti-HBs co-existence was similar to previous studies. Only one individual harbored mutation in the S gene a determinant associated with this profile. Little is known about this phenomenon; however, studies as ours may contribute for future enlightenment of this important issue. ***J. Med. Virol. 89:458–462, 2017.***

KEY WORDS: hepatitis B; HBsAg; antibody; mutation

INTRODUCTION

Approximately 240 million people are chronic carriers of the hepatitis B virus (HBV) [Lozano et al., 2012; World Health Organization, 2015a]. HBV infection varies greatly in different parts of the world, being designated as high, intermediate, and low endemicity [World Health Organization, 2015b]. A recent study reported that the overall prevalence in Brazil is less than 1%, with the exception of some areas in regions of the Amazon and rural regions in the Northeastern and Midwestern Brazil, where intermediate to high endemicities were already found [Souto, 2015].

Hepatitis B surface antigen (HBsAg) clearance and antibodies to HBsAg (anti-HBs) seroconversion involve the development of human immunity against the virus [Gerlich, 2007]. The presence of anti-HBs after vaccination has the same implication [Colson et al., 2007; Jang et al., 2009]. The concomitant

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presence of HBsAg and anti-HBs has been described since the 1970s, and this status presents a challenge for the clinical follow-up of HBV carriers. Furthermore, this profile may be of great epidemiological importance concerning the inactivity of anti-HBs antibodies after vaccination [Liu et al., 2012; Ding et al., 2016].

Co-occurrence of HBsAg and anti-HBs markers has been reported to vary between 2.43% and 8.9% worldwide [Lada et al., 2006; Colson et al., 2007; Zhang et al., 2007; Huang et al., 2010; Chen et al., 2011; Liu et al., 2012; Ding et al., 2015; Pancher et al., 2015; Pu et al., 2015; Ding et al., 2016; Liu et al., 2016].

The Pre-S/S regions of the viral genome encode HBV envelope proteins. The S protein, which corresponds to HBsAg, comprises the major hydrophilic region (MHR) (amino acids [aa] 99–169). The *a* determinant region is presented in the MHR (aa 124–147) and plays an important role in HBsAg structure because it maintains the immunogenicity of this protein [Zhang and Ding, 2015]. This determinant corresponds to the main site where HBsAg is recognized by anti-HBs antibodies, causing virus neutralization [Caligiuri et al., 2016].

The main hypothesis regarding the detection of concomitant HBsAg and anti-HBs is regarded to MHR mutation, particularly within the *a* determinant. Mutations in this region may cause relevant changes in the antigenic properties of HBsAg that reduce its susceptibility to the neutralizing action of anti-HBs immunoglobulin [Pondé, 2011; Shi et al., 2012; Brunetto, 2014].

Mutations in the *a* determinant occur mainly due to the following reasons: (i) immune pressure from HBV vaccination or as a result of human hepatitis B immuneglobulin (HBIG) administered to HBV carriers; and (ii) natural chronic infection response caused by the selective pressure of the immune system [Zhang et al., 2007; Pondé, 2011; Alavian et al., 2013; Coppola et al., 2015; Gao et al., 2015].

Fifty-four mutations presented in the *a* determinant are associated with the atypical profile of simultaneous presence of HBsAg and anti-HBs markers. Among these mutations, the G145R (glycine-to-arginine substitution at position 145 associated with hepatitis B vaccination and the use of HBIG) and I126S (isoleucine-to-serine substitution at position 126 related to chronic infection that alters the antigenicity of HBsAg, and immune escape) are the most frequently described until now [Cuestas et al., 2006; Lada et al., 2006; Lu et al., 2006; Mathet et al., 2006; Colson et al., 2007; Sayiner et al., 2007; Zhang et al., 2007; Velu et al., 2008; Jang et al., 2009; Hsu et al., 2010; Huang et al., 2010; van Dommelen et al., 2010; Wang et al., 2010; Chen et al., 2011; Liu et al., 2012; Hsu et al., 2013; Ding et al., 2015; Pancher et al., 2015; Pu et al., 2015; Ding et al., 2016; Liu et al., 2016].

In Brazil, the study of Bertolini et al. [2010] described 25 children that were born to HBsAg-positive mothers and received vaccination and/or HBIG. In their study, one child showed simultaneous positivity of HBsAg and anti-HBs, as well as detectable HBV DNA. Some mutations in S gene were observed, however, No G145R mutation was found [Carman et al., 1990].

Frequency of simultaneous HBsAg/anti-HBs is still scarce in specific regions. Therefore, we aimed to identify individuals with this profile among individuals of Maranhão, Brazil.

MATERIALS AND METHODS

Samples and Ethical Approval

A total of 3,984 blood samples of individuals from five municipalities (Humberto de Campos, Axixá, Morros, Icatu, and Urbano Santos) of Maranhão, Northeastern Brazil, were analyzed. These individuals were from a cross-sectional study performed to identify the prevalence of serological markers of Hepatitis B, C e Delta viruses (Data not published). Herein, we described the simultaneous occurrence of HBsAg and anti-HBs in the population of these municipalities. This study was approved by a research ethics committee and written consent was obtained from all individuals that agreed to participate of the study.

Serological Assays

All samples were evaluated for HBsAg, anti-HBs by enzyme-linked immunosorbent assay (ELISA) using commercial kits (Diasorin, Italy). The results for anti-HBs were obtained in absorbance and converted to IU/ml, with positive results defined as titers >10 IU/ml. The simultaneous HBsAg/anti-HBs samples were tested also for HBeAg and anti-HBe (Diasorin, Italy).

Samples of carriers with concomitant HBsAg and anti-HBs presence were submitted to more than one test for confirmation of this profile.

Amplification, Quantification, and Sequence Analyses

Viral load of the confirmed simultaneous HBsAg/Anti-HBs samples was determined and their viruses were subgenotyped. HBV DNA was extracted from 200 μl of serum or plasma using QIAamp DNA Blood Mini Kit (Qiagen®, Hilden, Germany) following the manufacturer's instructions.

The viral load was quantified using real-time PCR, as previously described [Sitnik et al., 2010].

To S gene sequence characterization, a fragment of 1,306 base pairs comprising part of Pre-S gene and the total S gene were amplified using a nested PCR protocol previously described [Barros et al., 2014; Gomes-Gouvêa et al., 2015].

Sequencing was performed using Sanger methodology by using the Big Dye Terminator kit v.3.1 and the primers (three pairs) previously described [Barros et al., 2014; Gomes-Gouvêa et al., 2015].

Electrophoresis was performed using an automatic sequencer (ABI 3500 DNA Sequencer; Applied Biosystems, Foster City, CA).

The quality of each sequence was evaluated using the Phred-Phrap software [Ewing and Green, 1998] and to assembly the consensus sequence of each sample was employed the software CAP3 available at the web page Electropherogram quality analysis Phred (http://asparagin.cenargen.embrapa.br/phph/). HBV subgenotypes were classified by comparing our sample sequences with references sequences retrieved from GenBank. Mutations in S gene were investigated throughout the MRH region between residues 100 and 169, which includes the *a* determinant (aa 124–147). The nucleotide sequences were aligned and translated to amino acids using the BioEdit software (v. 7.0.8) and the presence of mutations were identified by visual analysis.

RESULTS

Among the 92 samples found positive for HBsAg, only three presented HBsAg and anti-HBs co-existence. The epidemiological characteristics of these three carriers are described in Table I and serological and virological characteristics in Table II.

Only one viral sequence presented mutations in the S gene, within the *a* determinant. They were a thyrosine-to-asparagine substitution at position 134 and a threonine-to-isoleucine substitution at position 140 (Y134N/T140I) (Fig. 1).

DISCUSSION

Out of 92 individuals, three (3.26%) were identified with atypical serological profile and detectable HBV DNA among individuals from five municipalities of Maranhão, Northeastern Brazil. Similar frequency was already found worldwide, ranging from 2.43% to 8.9% [Lada et al., 2006; Colson et al., 2007; Zhang et al., 2007; Huang et al., 2010; Chen et al., 2011; Liu et al., 2012; Ding et al., 2015; Pancher et al., 2015; Pu et al., 2015; Ding et al., 2016; Liu et al., 2016]. Differences observed may be related to methodologies and different geographical regions.

TABLE I. Epidemiological Characteristics of the Carriers With Concomitant HBsAg and Anti-HBs

Carrier	A	B	C
Sex	Female	Male	Male
Municipality	Morros	Morros	Humberto de Campos
Area	Rural	Rural	Urban
Age (years)	62	30	61
Profession	Farmer	Farmer	Fisherman
Vaccine	Unknown	No	No
Sexual activity	Inactive	Active	Active
Stable relationship	One partner	One partner	More than one partner
Condom use	Never	Never	Sometimes
Shared needles or sharp instruments	No	Yes	Yes

TABLE II. Serological and Virological Characteristics of Individuals With Simultaneous HBsAg/Anti-HBs Positivity

Carrier	A	B	C
Anti-HBs (IU/ml)	18.1	260.6	100.4
HBV DNA (IU/ml)	1,306.57	1,176.4	91,641.4
Anti-HBc	+	+	+
Anti-HBe	+	+	−
HBeAg	−	−	−
Subgenotype	D4	D4	D4
Mutations	Y134N/T140I	ND	ND

ND, not detected.

Concomitant positivity of HBsAg and anti-HBs markers has been attributed to selection of HBV variants that escape anti-HBs activity. Reports have shown that modifications in amino acids of *a* determinant region, due to mutation in S gene of HBV, may alter the S protein structure conformation, and thus, leading to immune escape [Lada et al., 2006; Ding et al., 2016]. Occurrence of these mutations in certain population groups is determined by factors that can promote the emergence of HBV immune escape such as endemicity, vertical transmission, and vaccination [Zaaijer et al., 2008; Lazarevic et al., 2010].

Among the individuals with atypical serological profile in this study, only one individual presented mutations Y134N and T140I. The first was already found in individuals with simultaneous positivity for HBsAg and anti-HBs [Li et al., 2009; Pu et al., 2015]. Furthermore, Y134N was also identified in HBV carriers with resistance mutations to nucleoside and nucleotide analogs in liver transplant recipients with occult hepatitis B [Rahimi et al., 2015], and in individuals with occult Hepatitis B and hepatocellular carcinoma [Pollicino et al., 2007]. Therefore, this is not a specific mutation of atypical profile of concomitant HBsAg and anti-HBs positivity; however, it may have probably its role. More studies are necessary to understand this phenomenon. T140I was also described in Chinese chronic carriers of HBV with concurrent HBsAg and anti-HBs [Ding et al., 2015]. Immunized children born to HBsAg-positive mothers also harbored this amino acid change [Hsu et al., 2010; Hsu et al., 2013; Jaramillo and Navas, 2015] as well as chronic carriers of HBV with cirrhosis or hepatocellular carcinoma [Yamani et al., 2015], carriers of occult hepatitis B [Huang et al.,

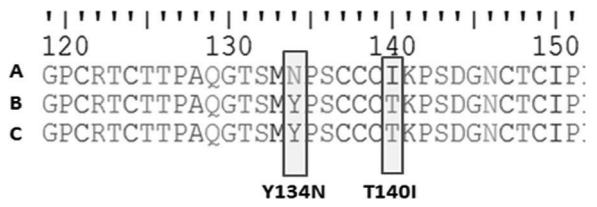

Fig. 1. Amino acids sequences of the *a* determinant region of HBsAg extracted from sequence alignment. The columns show the regions with mutations.

2012; Lazarevic, 2014], and HBV carriers who did not receive the vaccine or HBIG [Komatsu et al., 2012].

In this study, the substitution of glycine (G) by arginine (R) at position 145 (G145R) in the *a* determinant of S protein, which is the most commonly variant associated with vaccine-induced resistance and HBIG, was not found. Actually, we lacked information about vaccination of the individuals harboring these mutations in our study.

Concerning the concomitant presence of HBsAg and anti-HBs, only one study has been reported in Brazil [Bertolini et al., 2010]. They did not report the G145R mutation in their study but a substitution in threonine (T) at position 140 and glutamine (Q) at position 129 within the *a* determinant, as well as others in the S protein. These mutations may also affect the immunogenicity of the vaccine and HBIG.

This study reported two individuals with atypical profile of HBsAg and anti-HBs positivity who did not present mutations in the *a* determinant of the S gene. This finding can be explained by mutations in other regions not examined in this study as the Pre-S/S region. Mutations in Pre-S/S region of HBV genome have already been described in individuals with this anomalous profile [Chen et al., 2011; Pu et al., 2015; Liu et al., 2016], such as L209V [Mathet et al., 2006].

In conclusion, the frequency of individuals with atypical profile of HBsAg and anti-HBs positivity was similar to previous studies described worldwide. Little is known about this phenomenon, however, studies as our may contribute for future enlightenment of this important issue.

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5.4 CAPÍTULO IV - Artigo 4- High Prevalence of Hepatitis B Subgenotype D4 in Northeast Brazil: an Ancient Relic from African Continent?

5.4.1 Nome do Periódico com sua classificação na WEBQUALIS da CAPES (A1, A2, B1 ou B2) na área de Avaliação Medicina I- Annals of Hepatology (B1)

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ORIGINAL ARTICLE

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High Prevalence of Hepatitis B Subgenotype D4 in Northeast Brazil: an Ancient Relic from African Continent?

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ABSTRACT

Introduction. Hepatitis B virus (HBV) infection leads to a chronic liver disease that is distributed worldwide. The characterization of HBV into genotypes/subgenotypes is not only a mere procedure for distinguishing different HBV strains around the world because determining their geographic distribution is crucial to understanding their spread across the world. **Material and methods.** We characterized different HBV genotypes and subgenotypes in five municipalities located in northeastern Maranhão, in the Brazilian north Atlantic coast. 92 HBsAg-positive individuals were submitted to PCR (polymerase chain reaction). Fifty samples were sequenced using automated Sanger sequencing and classified by phylogenetic methods. **Results.** Subgenotypes D4 and A1 were found in 42 (84%) and eight (16%) samples, respectively. To our knowledge, this is the first study to describe a high frequency of subgenotype D4 in any population. Subgenotype A1 is frequently found across Brazil, but D4 has been rarely detected and only in a few Brazilian states. This study shows the characterization of HBV subgenotypes from a population based study in the state of Maranhão, particularly in populations that do not have frequent contact with populations from other regions of the world. **Conclusion.** Our findings showed a HBV subgenotype profile that probably reflect the viruses that were brought with the slave trade from Africa to Maranhão. This study also reinforces the need to evaluate the status of HBV dispersion not only in large urban centers, but also in the hinterland, to enable the implementation of effective control and treatment measures.

Key words. HBV. Genotype. Slave trade. Maranhão.

INTRODUCTION

Even though an efficient vaccine to prevent infection from the hepatitis B virus (HBV) is available since the 1980s more than 700,000 people have died worldwide from its complications in 2010.[1,2] HBV is a highly prevalent and silent infection.[3] HBV infection is a public health problem in regions where complete vaccination coverage has been difficult to achieve, particularly in infants, who are more prone to develop the chronic course of the disease.[4,5]

East Asia and Sub-Saharan Africa show high HBV prevalence, whereas Tropical Latin America, Central Latin America, Western Europe, and North America are considered low prevalence areas.[6] According to a national survey, Brazil is considered a low prevalence area,[7] although some areas with higher prevalence have been reported in the Amazon Basin and in the Southeast and South regions.[8]

HBV is a DNA virus classified into eight genotypes (A-H) that differ by 7.5% or more in their genomic sequence and have distinct geographical distributions.[9,10] A putative genotype I has also been described,[11] although some au-

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thors have contested it, based on new recommendations for sorting a new genotype.[12,13] Additionally, a genotype J has also been described in Japan.[14] This tentative genotype shows similarities to gibbon and orangutan viruses and further studies are needed to ratify its classification.[15] HBV genotypes may also be further divided into subgenotypes based on a nucleotide intragenotypic difference between 4 and 7.5%.[9]

The classification of HBV into genotypes/subgenotypes is not only a mere procedure for distinguishing different strains around the world because determining their geographic distribution is crucial to understanding their spread and analyzing evolutionary pathways.[16,17]

The distribution of genotypes around the world is a reflection of human population movements and is related to each ethnical background.[15,18] In Brazil, genotype A is the most frequent genotype found followed by D, and F,[19-26] whereas A1, D3, and F2a seem to be the most prevalent subgenotypes.[19-22,24,27]

The population of Brazil is composed of three main ancestral backgrounds: Europeans, Amerindians, and Africans.[28] The latter group consisted of African slaves brought to work in many regions of the New World. Brazil received the largest numbers of slaves, followed by the Caribbean.[29] Maranhão is a state in the north Atlantic Coast of Brazil that has particular features in the history of African slave trade: later establishment of slavery, isolation from other regions of the country due to the lack of river connection with the continent, and the similarity of slave trading routes with those in North Atlantic regions.[30]

The state of Maranhão is one of the Brazilian states with a higher percentage of Afro-descendants and remained isolated from the rest of the country until the mid-19th century.[30] There are only two previous studies analyzing the distribution of HBV genotypes in the state of Maranhão: one that analyzed patients followed up in the city of São Luís, the state capital,[19] and another that analyzed a quilombo community in northwestern Maranhão.[31] In both studies, HBV genotypes also found in Africa were the most frequent, but genotype D4 found at the São Luís Referral Center for Liver Studies, São Luís, Maranhão, Brazil, has rarely been detected in other Brazilian regions.

This study characterized two different HBV subgenotypes found in five municipalities located in northeastern Maranhão.

MATERIAL AND METHODS

Sample selection and ethical approval

A total of 3,860 samples were screened for HBsAg serological marker using commercial kits according to the manufacturer's instructions (DiaSorin, Italy) in the five municipalities located in northeastern Maranhão along the Brazilian north Atlantic Coast (Figure 1) from 2012 to 2014. The representative sample number for the study region was calculated by considering an HBsAg prevalence of 0.5% in Brazil and the total number of individuals in each municipality. The collection procedure was per-

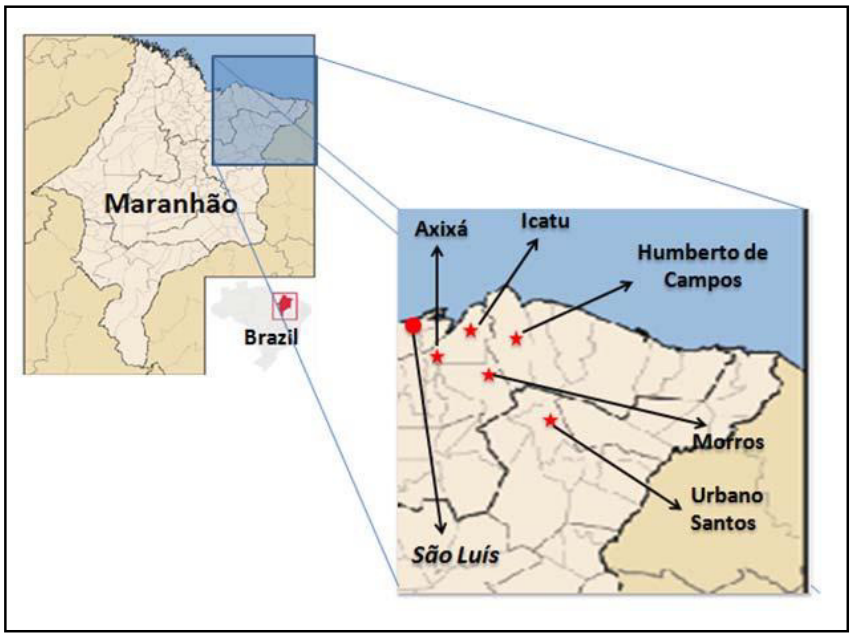

Figure 1. *Geographic location of the five municipalities sampled in northeastern Maranhão, Brazil. Each star represents one municipality. The red circle indicates de state capital, São Luis.*
Source. Modified from: Wikimedia Commons -https://commons.wikimedia.org/wiki/File:Maranhao_MesoMicroMunicip.svg#/media/File:Maranhao_MesoMicroMunicip.svg

formed by two-stage cluster sampling: first by choosing sectors according to population proportion and second by selecting the squares in these sectors by random sampling.

The study was approved by the research ethics committee at University Hospital, Federal University of Maranhão (HUUFMA), São Luís, Maranhão, Brazil, and written consent was obtained from all individuals that agreed to participate in the study.

HBV DNA amplification and sequencing

HBsAg positive samples were analyzed by nested polymerase chain reaction (nested PCR) to detect HBV DNA. HBV DNA was extracted using the QIAamp DNA Blood Mini kit (Qiagen®, Hilden, Germany) following the manufacturer's instructions. Fragments of 1,306 base pairs (bp) comprising the whole HBsAg and part of the DNA polymerase genes were amplified by nested PCR. Primers' sequences, PCR conditions, and sequencing procedures are described elsewhere.[19,27] All procedures were strictly done following measures for avoiding contamination as well as negative and positive controls were used in PCR assays.[32]

Viral load

PCR negative samples were analyzed for detection of viral load by real time PCR. We used the TaqMan® system (Life Technologies, Carlsbad, CA, USA) and probes, primers, and thermocycling conditions used are described elsewhere.[33] The amplification was performed using a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, USA).

HBV genotyping and subgenotyping

Chromatograms were evaluated using Phred-Phrap software[34,35] and a quality score of 20 was used to select good quality readings. The consensus sequence of each sample was obtained using CAP3 software available at the Electropherogram quality analysis webpage (http://asparagin.cenargen.embrapa.br/phph/). Sequences were aligned using BioEdit (v. 7.0.8) software and edited using CLUSTAL W software.[36] HBV genotypes and subgenotypes were classified by phylogenetic reconstructions with published reference sequences from the GenBank database (http://www.ncbi.nlm.nih.gov/).

Phylogenetic trees were constructed using the Bayesian Markov Chain Monte Carlo (MCMC) method implemented in the BEAST package v.1.6.1[37] under a relaxed molecular clock using GTR + G + I as nucleotide substitution model; MCMC was run for 20 million generations and trees were sampled every 2000 generations. Maximum clade credibility tree was summarized using TreeAnnotator v.1.6.1 and the tree was visualized in FigTree v1.4.2. software (available at: http://tree.bio.ed.ac.uk/software/figtree).

Serotypes were sorted according to the position of amino acids in the S gene by visual analysis.[10]

All sequences were submitted to GenBank under accessions numbers KX302085 - KX302134.

Statistical analysis

The Student's t-test was used to determine the difference in mean age between subgenotypes; the Fisher's exact test was used to compare the other variables between subgenotypes. Differences were considered significant at $p < 0.05$. All analyses were performed using STATA software version 11.0.

RESULTS

Demographic characteristics of HBV-DNA positive individuals

Of the 3,860 samples, 92 were positive for HBsAg serological marker. Fifty-one (55.4%) of the 92 HBsAg-positive individuals had detectable HBV DNA by nested PCR. Most HBV-DNA positive individuals were men (56.9%; 29/51) and their mean age was 44.3 (SD = 21.1) years. In addition, most HBV-positive samples were from Humberto de Campos (43.1%; 22/51). We also found that most HBV-positive individuals were from the rural zone (70.6%; 36/51), had a family income of < 1 minimum wage (64.7%; 33/51), and declared themselves as being Afro-American or mestizo/mullato (76.5%; 39/51).

Viral load

Forty-one of the 92 positive HBsAg samples were negative for HBV-DNA. These negative samples were submitted to viral load detection which was performed in 39 of the 41 samples because two serum samples were unusable. In total, 36 of the 39 samples analyzed had undetectable HBV viral load confirming our findings by nested PCR.

Genotype/subgenotype and serotype distribution

All 51 positive samples had their partial HBV sequence determined. One sample was excluded from the final analysis because the sequence did not achieve a good quality

Table 1. Demographic characteristics of HBV-carriers and subgenotypes found in this study.

Variable	Subgenotype (%) A1	D4	Total	P
N	8 (16)	42 (84)	50	
Male	5 (17.86)	23 (82.14)	28 (56)	1
Female	3 (13.64)	19 (86.36)	22 (44)	
Mean age (± SD)	41.5 (±18.7)	45.3 (±21.8)	44.7 (±21.2)	0.647
Origin				
Axixá	1 (50)	1 (50)	2 (4)	**0.015**
Morros	2 (16.7)	10 (83.3)	12 (24)	
Icatu	4 (50)	4 (50)	8 (16)	
Humberto de Campos	1 (4.55)	21 (95.4)	22 (44)	
Urbano Santos	0	6 (100)	6 (12)	
Sector				
Urban	3 (21.4)	12 (78.6)	15 (30)	0.683
Rural	5 (13.9)	30 (86.1)	35 (70)	
Ethnic group*				
Afro-American	3 (25)	9 (75)	12 (24)	0.32
Caucasian	0	5 (100)	5 (10)	
Native American	2 (40)	3 (60)	5 (10)	
Mestizo/Mulatto	3 (11.5)	23 (88.5)	26 (52)	
Other/unknown	0	2 (100)	2 (4)	
Education level				
No education	0	12 (100)	12 (24)	0.336
Incomplete Elementary education	5 (23.81)	16 (76.2)	21 (42)	
Complete Elementary education	0	4 (100)	4 (8)	
Incomplete High school education	1 (20)	4 (80)	5 (10)	
Complete High school education	2 (28.6)	5 (71.4)	7 (14)	
Higher education	0	0	-	
Not applied	0	1 (100)	1 (2)	
Family Income				
< 1 mw**	2 (6.3)	30 (93.7)	32 (64)	
1-3 mw	6 (33.3)	12 (66.7)	18 (36)	**0.019**

** Self-declaration. ** Minimum wage.*

index, but the remaining 50 samples had their HBV genotype and subgenotype determined (Table 1).

Genotypes A and D were only found in our sample and genotype D was the most frequent (84%; 42/50). The phylogeny showed that all genotype A sequences were subgenotype A1 (adw2) and all genotype D individuals were subgenotype D4 (ayw2). Additionally, subgenotype D4 clustered in a monophyletic group together with other sequences from Maranhão, whereas subgenotype A1 samples grouped with different HBV/A1 branches, except with the one containing sequences from Africa only (Figure 2).

HBV subgenotypes and sociodemographic characteristics

We found no significant difference in most sociodemographic characteristics between subgenotypes A1 and D4, except for municipality and family income (Table 1). There were more subgenotype D4 individuals in Morros, Humberto de Campos, and Urbano Santos than in Axixá and Icatu (P = 0.015). Additionally, the frequency of subgenotype D4 was higher in families with income < 1 minimum wage (P = 0.019).

DISCUSSION

Up to the present time, this is the third study to determine HBV genotype/subgenotype distribution in the state of Maranhão, in the north Atlantic coast of Brazil[19,31] but it is the first to obtain HBsAg-positive samples from representative number of individuals of a regional population-based survey. The study region is located in northeastern Maranhão and consists of five municipalities with low socio-economical index.[38]

Most infected individuals lived with less than one minimum wage for the whole family and the level of education was often low. Thus, because of poverty and low educational status, these individuals are often unaware about means of transmission and prevention of diseases such as hepatitis, and thus may be more susceptible to in-

Figure 2A. *Phylogenetic analysis of HBV subgenotype isolates from different regions of Maranhão state, Brazil, detailing subgenotypes A1* ***(A)*** *and D4* ***(B)****. Analyses were performed by Bayesian Inference using the Bayesian Markov chain Monte Carlo (MCMC) method. Sequences identified in this study are highlighted (#) and reference sequences from GenBank are indicated by their corresponding genotype/subgenotype accession number and origin. The posterior probability values are shown for key nodes.*

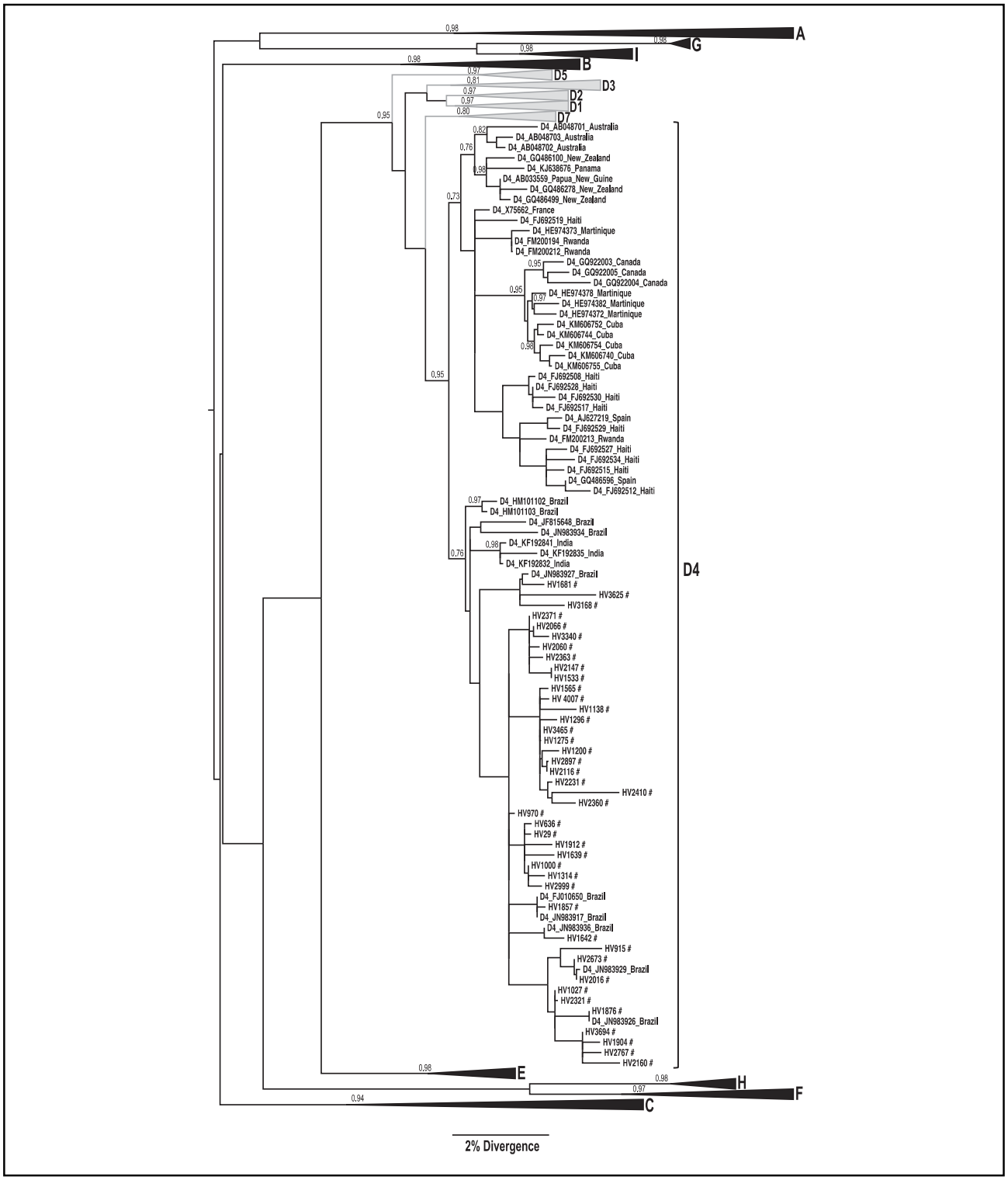

Figure 2B. *Phylogenetic analysis of HBV subgenotype isolates from different regions of Maranhão state, Brazil, detailing subgenotypes A1* ***(A)*** *and D4* ***(B)****. Analyses were performed by Bayesian Inference using the Bayesian Markov chain Monte Carlo (MCMC) method. Sequences identified in this study are highlighted (#) and reference sequences from GenBank are indicated by their corresponding genotype/subgenotype accession number and origin. The posterior probability values are shown for key nodes.*

fection. The delayed improvements in living and hygiene conditions and the difficulties in launching prevention, control, and treatment strategies that are important to control viral hepatitis and other diseases are known and have been described elsewhere.[8]

Two different HBV genotypes were found in our sample. Genotype D was the most prevalent followed by few genotype A cases. This finding is contrary to the HBV genotype prevalence described for Maranhão and Brazil as a whole, where genotype A is the most prevalente.[19,21-26,31,39] An exception to that pattern is found in southern Brazil, where genotype D is the most frequent genotype,[20,40,41] which is related to the immigration of European people from highly HBV/D prevalent regions.[42]

Only two subgenotypes were identified in our study, A1 and D4 being the latter the most frequent. Subgenotype A1 is the most frequent subgenotype found in cases of genotype A across Brazil[27] and it was also the most frequent subgenotype found in the previous study conducted in São Luís, in Maranhão.[19] In addition to subgenotype A1 that study also identified other four subgenotypes (D2, D3, F2a, and D4). Unlike the current study, subgenotype A1 was the most prevalent in that study, even though subgenotype D4 also had a relatively high frequency (24%; 29/119).[19] The difference between our results and those of Barros, *et al*. (2014) is noteworthy and may be related to the samples analyzed: the population from the latter study consisted of chronic HBV carrier patients treated at a referral center for treatment of liver disease, and even though it covers the entire state of Maranhão, more than 75% of participants were from the metropolitan area of São Luís, the state capital. Thus, subgenotype A1 seems to be more prevalent in the metropolitan region, whereas subgenotype D4 is more frequent in other regions of the state such as the northeast.

Alvarado-Mora, *et al*. (2011) only found subgenotype A1 in four sequenced samples from quilombo Frechal, located in the municipality of Mirinzal, northwestern Maranhão.[31] More studies in other regions of Maranhão should be conducted to analyze a larger number of HBV-positive samples for a better understanding of subgenotype prevalence in this region.

Subgenotype D4 was previously found in the aforementioned study conducted in Maranhão,[19] but it is not frequently found in Brazil. This genotype was initially described in two different studies conducted in the Brazilian Amazon, in the states of Amazonas[21] and Rondônia.[24] Genotype D4 was also found in 5.1% (36/702) of samples collected across Brazil,[27] mostly in Maranhão, but also in other states in the southeast (São Paulo and Minas Gerais) and north (Pará) of Brazil.[43] We hypothesize that internal migrations among different Brazilian states may explain the presence of subgenotype D4 in these regions, particularly in Pará, which has a large border with the state of Maranhão.

The high prevalence of subgenotype D4 in Maranhão supports the hypothesis that this strain was not introduced in Brazil through European immigration because this subgenotype is not frequent in Europe.[42] Zehender, *et al.* (2012) performed for the first time a HBV-D epidemiological reconstruction by phylodynamics and phylogeographic analysis in Europe.[44] Although they did not included D4 sequences in their analysis, a preliminary review of them showed that D4 and D7 subgenotypes are close related. Furthermore, other study also found this relation between these two subgenotypes.[45] HBV-D7 is more often restricted to the Northern Africa[46,47] and may give support to the hypothesis of HBV-D4 distribution from this continente.[19]

In fact, subgenotype D4 has been previously described in many countries worldwide.[10,48-55] HBV D4 has also been found in Caribbean countries of significant African descent such as Haiti, Martinique, and Cuba.[56-58] Interestingly, in Kenya, only subgenotypes A1 (85.7%) and D4 (14.3 %) have been reported,[49] but in proportions that are the opposite of those found in Maranhão. As suggested by Barros, *et al.* (2014), subgenotype D4 may have infected individuals frequently in the past, and during the period of slavery these strains may have reached other continents where African slaves were also traded.[19] For instance, Haiti and Cuba are regions outside Africa with a high frequency of subgenotype D4 cases.[56,58] The Caribbean had intense trading of slaves from Africa, lower only than that in Brazil.[29] Thus, the history of population formation, combined with molecular evidence of virus traits, may support a better understanding of the spread of infections such as HBV.

Subgenotype D4 strains found in Brazil clustered in a separate branch in the phylogenetic tree with other samples described elsewhere (except from three from India), suggesting the introduction of a unique strain in the country. It is noteworthy that only these India sequences appeared to be related to our sequences apart from any other described in the world. These Indian strains are from Tripura State which is the first region in India where subgenotype D4 was found.[53] Although the authors did not found a high prevalence of the HBV-D4 (13/76; 17.1), Portuguese vessels might have brought from India some strains of this infection to Maranhão, mainly when British begun to suppress the Atlantic slave trade and forced the Northern Atlantic traders to change their routes to South-East Africa.[30,53,59,60] Subgenotype A1 strains also clustered in a branch different than the one where most African sequences were grouped and differently than subgenotype D4 strains, A1 strains did not aggregate in a few branches but rather in many branches containing samples from across the world, with only few African samples from So-

malia and South Africa. Thus A1 strains cannot be considered an African clade. In a study that analyzed A1 strains from Brazil,[60] the authors concluded that Brazilian A1 genotypes are not of Central or Western Africa origin, but originated from slaves brought from southeastern Africa during the mid-19th century, because the study's samples clustered into an "Asian-American" clade similarly to the samples in our study. However, there are few available sequences with these genotypes from East African countries, despite the reports of slaves coming to Maranhão from these countries.[61]

The Brazilian population was formed by three ancestral backgrounds in decreasing order of contribution: Europeans, Africans, and Amerindians. Nevertheless, the state of Maranhão is one of the regions in Brazil with the greatest contribution of African ancestry.[28] Our results show that most individuals who were assigned to subgenotypes A1 and D4 declared themselves as being Afro-American (24%) or mestizo/mulatto (52%), reinforcing the association between these two subgenotypes and African ancestry. Moreover, the pattern of slavery establishment in Maranhão was peculiar: due to geographic features (isolation from other regions in the country because of a lack of river connection to the continent) and navigation issues (wind and ocean currents), slave trade routes in Maranhão were more similar to those in Caribbean and North Atlantic countries than those in the rest of Brazil.[30] Thus, we expected to find the same subgenotype in Maranhão and in Caribbean countries, which is not the same one found in Bahia, another Brazilian state with a large number of Afro-descendants.[27] Finally, the recent finding of HDV-8 in Maranhão co-infecting individuals that carried HBV/D4 corroborates the African origin of the latter subgenotype.[62,63]

This study emphasized the presence of HBV genotypes that might be originated from Africa in the state of Maranhão, particularly in populations that do not have frequent contact with populations from other regions of the world. This pattern probably reflects the viruses that were brought with the slave trade from Africa to Maranhão that was interrupted in the middle of the 19th century. Maranhão has a great contribution of African ancestors originated from a particular slave trade route that is similar to the one followed in the Caribbean islands and both areas share a similar pattern of HBV subgenotype distribution.

This study has unexpectedly shown a low prevalence of HBV DNA among the HBsAg individuals. This may be explained by the nature of the sample. The individuals who participated of this study were from an epidemiologic survey rather than individuals searching for medical care. However, the high prevalence of HBV D4 strain may scatter light on the interaction of this virus and the human host, that is, genotype D4 may influence the immunologic state of HBsAg marker.[64] Only with more studies related to this issue with follow up of patients from this population would explain this finding.

This study also reinforces the need to evaluate the status of HBV dispersion not only in large urban centers, but also in the hinterland, to enable the implementation of effective control and treatment measures. Studies involving population samples from wide geographic areas and hard-to-access regions are difficult to perform, mainly due to the high costs and lack of specialized human recourses. Nevertheless, further investigations in isolated areas are needed because a better knowledge of HBV infection across the world is essential for the effective control of this infectious disease.

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6. Considerações Finais e conclusão

Este estudo apresenta limitações e pontos fortes. O principal ponto forte foi a amostra representativa destes municípios, uma vez que a amostra principal foi calculada usando uma prevalência de 0,5% para o HBsAg, quase um quinto da prevalência real, permitindo uma segura análise da prevalência e dos fatores de risco. Entre as limitações estão todas aquelas decorrentes de um estudo transversal, além de um possível viés de informação sobre o comportamento sexual e o uso de drogas, já que as informações foram coletadas em entrevista única durante visita domiciliar. Precauções como o treinamento dos entrevistadores e a realização de entrevistas individuais foram tomadas para tentar minimizar estas limitações, mas é possível que não tenham sido suficientes para evitar distorções.

Os achados apresentados neste estudo reforçam a heterogeneidade da prevalência fora das capitais brasileiras, sugerindo fatores associados com a transmissão do vírus B, mostrando a necessidade de melhorar o emprego de estratégias de grande impacto já implementadas, como a imunização de crianças, adolescentes e adultos, além das estratégias de rastreio de infectados e prevenção de transmissão vertical, considerando a implementação de novas políticas para populações de risco, como os usuários de drogas ilícitas. Reforçam, também, a necessidade de novas pesquisas para determinar a real prevalência da infecção pelo vírus D.

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8 APÊNDICE A - Questionário sócio-demográfico

ESTUDO DAS HEPATITES B, C E D NOS MUNICÍPIOS DE URBANO SANTOS, HUMBERTO DE CAMPOS E DA REGIÃO DO BAIXO MUNIM, MARANHÃO, BRASIL.

Ficha n.º _____ Data do preenchimento ___/ ___/ ___
Município_____ Zona _____
Nº setor censitário _____

I. IDENTIFICAÇÃO		
Nome: ***Apelido:***		
2. ***Data de nascimento*** ____/____/____ ***Idade***:		Idade:
3. ***Sexo***: (1)Masculino (2) Feminino		Sexo:
4. Grau de instrução (0) Analfabeto (1) Ensino Fundamental Incompleto (2) Ensino Fundamental Completo (3) Ensino Médio Incompleto (4) Ensino Médio Completo	 (6) Ensino Superior Incompleto (7) Ensino Superior Completo (8) Não se aplica (9) Não quer responder	Grau de instrução:
5. ***Local de nascimento:***		Lnascimento:
6. ***Residência atual:***		Resdatual:
Mora há quanto tempo:		Moratempoa;
Ponto de referência:		
7. Última procedência:		Utimaproa;
8. Outros locais onde morou/trabalhou:		Loctrabalhou:
9. Há quanto tempo morou/trabalhou		Tempotrabalhou:
10. Cor (impressão do entrevistador (1) Preta (2) Branca (3) Marrom (4) Vermelha (5) Amarela (6) Outra:		Cor:
11.Religião (0) Nenhuma (1) Católico (2) Evangélica (3) Outra:		Religião:
12.Estado civil (1) Solteiro (2) Casado (3) Separado judicialmente (4) Divorciado (5) Viúvo (6) União estável/mora junto		Estado civil:
II – OCUPAÇÃO		
13. Atual:		Atualocupação:
14. Pregressa:		Pregressocupação:

III - RENDA FAMILIAR	
15. Renda familiar em salários mínimos: (0) menos de 1 salário mínimo; (1) 1 a 3 salários mínimos; (2) 4 a 6 salários mínimos; (3) 7 a 11 salários mínimos; (4) mais de 11 salários mínimos	Rendafam:
IV - IMUNIZAÇÃO	
16. Cartão de vacina: (1) Sim (2) Não (3) Não sabe	Cartvacina:
17. Vacinação para hepatite B (1) Sim (2) Não (3) Não sabe Quantas doses? (1) 1 dose (2) 2 doses (3) 3 doses	VacinaHB: VacHBdose:
18. Fez anti-Hbs (Teste para Hepatite pós-vacina)? (1) Sim. *Se sim, há quanto tempo?* ( ) (2) Não	AntiHbs: tempAntiHBS:
19. Doador* (1) Sim. *Se sim, n^{o} doações por ano: ( )* (2) Não (3) Não se aplica	Doador: Anodoador:
20. Última aceita: _____/_____/_____	Ultimaaceita:
21. Transfusão sanguínea (1) Sim (2) Não ***22. Se sim, data da última transfusão:*** ***23. Local da ultima transfusão:***	Transfussangue: Datatransfussangue: Localultimasangue:
VI – DROGAS	
24. Álcool: (1) Sim (2) Não (3) Não se aplica	Alcool:
Tempo de Consumo (anos): ***Frequência:*** (1) diariamente (2) semanalmente (3) esporadicamente	Alcooltemp: Alcoolfreq:
Quantidade (*fechar em número exato/copos*): _____ **Tipo de bebida**:_____ **Abstinência há** (meses):_____	Alcoolquant: Alcooltipo: Alcoolabst:
25. Drogas Ilícitas (1) Usa (2) Já usou	Drogailic:

(3) Nunca usou (4) Não respondeu (8) não se aplica	
26. Via: (1) Inalatória (2) Endovenosa	Dorgavia:
27. Seringa própria: (1) Sim (2) Não	Drogaseringaprop:

28. Tipo de droga:	Drogatipo:
VII – ATIVIDADE SEXUAL	
29. Vida sexual: (0) Nunca teve (1) Ativa (2) Inativa (9) Não quer responder (3) Não se aplica	Vidasex:
30. Prática sexual (pregressa e atual) (1) Homem/Mulher (2) Homem/Homem (3) Mulher/Mulher (4) Mulher e Homem (bissexual)	Pratsex:
31. Relação sexual estável: (1) 1 parceiro (2) Mais de 01 parceiro (9) Não quer responder	Sexestavel:
32. Numero de parceiros *(+ 3 parceiros em 6 meses)*: (1) Sim (2) Não (9) Não quis responder	Sexparceirnumero:
33. Preservativo: (1) Sempre (2) Às vezes *(3)* Nunca (9) Não quer responder	Sexpreserv:
Em caso positivo, usa preservativo para : (1) Proteção contra DST (2) Contraceptivo (3) Não sabe	Sexpreservpara:
VIII-PACIENTE SUBMETIDO A:	
Endoscopia: (1) Sim (2) Não ***Qts vezes***: Local:	Endoscopia: Endoscopiavezes: Endoscopialocal:
Cirurgia: (1) Sim (2) Não ***Qual***_____ Há quanto tempo (anos):	Cirurgia: Cirurgiaqual: Cirurgiatemp:
Tratamento Odontológico: (1) extração (2) extração + canal	Tratodonto:

(3) Canal (4) Nunca fez (5) Outro: _____ Há quanto tempo fez o tratamento (anos): _____ Foi com odontólogo (1) Sim Qnd?:_____ Local _____ 4	Tratodontotemp: Odontologo: Odontologoqnd: Odontologoloc: Protetico:
Foi com protético (1) Sim Qnd?:_____ Local _____	Proteticoqnd: Proteticoloc:
Tatuagem (1) Sim. Qnd?:_____ Local _____ (2) Não	Tatuagem: Tatuagemqnd: Tatuagemloc:
Piercing (1) Sim. Qnd?:_____ Local _____ (2) Não	Piercing Piercingqnd: Piercingloc:
Acupuntura (1) Sim. Qnd?:_____ Local _____ (2) Não	Acump: Acumpqnd: Acumploc:
Injeções com seringa de vidro e agulha de metal no passado antes 1993? (1) Sim (2) Não (3) não lembra	Seringvidro:
IX-PERFURO-CORTANTES (Materiais usados)	
Alicate: (1) No salão (4) Salão-individual (2) Salão-compartilhada (5) Casa-individual (3) Casa-compartilhada. (6) Outros materiais: ***Barbeadores*** (1)No salão (3) Casa-individual (2) Casa-compartilhada ***Navalha*** (1) No salão (3) Casa-individual (2) Casa-compartilhada ***Pinça depilatória*** (1)No salão (2) Casa-individual (3) Casa-compartilhada	Alicate: Barb: Navalha: Pinça:
X- EXPOSIÇÃO A MATERIAL BIOLÓGICO	
Lesão de pele (ferimento, perfuração) em acidente, no trabalho, etc.	Lesão:

	(1)Sim (2)Não (3) Não lembra	Lesãoqnd:
Qdo	Qts vezes?	Lesãovezes:
XI-ANTECEDENTES PESSOAIS		
HIV (1)Sim (2)Não (3) Não sabe	***IRC*** (1)Sim (2)Não (3) Não sabe	Hiv:
		Dst:
DST´S (1)Sim (2)Não (3) Não sabe	***Hepatite B*** (1)Sim (2)Não (3) Não sabe	Icter:
		Irc:
Icterícia (1)Sim (2)Não (3) Não sabe	***Hepatite C*** (1)Sim (2)Não (3) Não sabe	HpB:
		HpC:
	Outros:	Outroantecp:
XII – ANTECEDENTES FAMILIARES		
Contato com portadores de Hepatite B-C (1)Sim (2)Não		ContatoHpBC:
Quem era o portador (1) pai (4) mãe (2) Filhos (5) irmãos		PortHpBC:
. (3) esposo(a)/companheiro(a) (6) não domiciliar		
O portador apresentou (1) Icterícia (2) IRC-Diálise (3) Outros_____		Portapresent: Outroantfam:
XIII – ADULTO/ADOLESCENTE (SE SEXO FEMININO)		
Gestante: (1)Sim (2)Não Se sim, mês de gestação: Realiza pré-natal? (1)Sim (2)Não		Gest: Gestmes Prenatal
Nutriz: (1)Sim (2)Não GESTA: _____ PARA: _____ ABORTO: _____ Quando ocorreu o último parto? _____ Onde? Tipo de parto? (1) Vaginal (2) cesárea		Nutriz: Gesta: Para: Aborto: Ultimopart: Ondeparto: Tipoparto:
XIV – DE QUAL RAÇA VOCÊ SE CONSIDERA?		
(1) Negra (2) Branca (3) Indígena (4) Mestiça (5) Outra. Qual? _____ Possui parentesco estrangeira conhecida? (1)Sim (2)Não **De que origem você se considera?** (1) Européia (2) Africana (3) Ameríndia (4) outra		Raça: Outraraça: Origem:

ANEXO A – PARECER CONSUBSTANCIADO DO CEP

HOSPITAL UNIVERSITÁRIO DA UNIVERSIDADE FEDERAL DO MARANHÃO/HU/UFMA

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Estudo das Hepatites B, C e D nos Municípios de Urbanos Santos, Humberto de Campos, e da Região do Baixo Munin, Maranhão, Brasil.

Pesquisador: Adalgisa de Souza Paiva Ferreira

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 1

CAAE: 20935413.5.0000.5086

Instituição Proponente: Hospital Universitário da Universidade Federal do Maranhão/HU/UFMA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 448.731

Data da Relatoria: 01/11/2013

Apresentação do Projeto:

A hepatite viral é uma doença provocada por diferentes agentes etiológicos que apresentam particularidades importantes em suas características epidemiológicas, clínicas e laboratoriais. A Organização Mundial da Saúde (OMS) estima que 350 milhões e 170 milhões de pessoas estejam infectadas com os vírus das hepatites B (HBV) e C (HCV), respectivamente. A infecção pelo vírus da hepatite D (HDV) tem distribuição geográfica heterogênea, estimando-se cerca de 18 milhões de infectados pelo HDV entre os 350 milhões de portadores crônicos do HBV. Estudo transversal com 4000 participantes para conhecer a prevalencia de hepatites virais e caracterizar os genótipos e subgenótipos desses vírus nos municípios de Urbano Santos, Humberto de Campos e de municípios da região do Baixo Munim (Axixá, Morros e Icatú)-MA. Serão submetidos à coleta de 15 ml de sangue periférico para realização dos seguintes marcadores sorológicos: HBsAg, anti-HBc e anti-HBs, anti-HDV e anti-HCV que serão realizados no Laboratório Central do Estado do Maranhão (LACEN), através da técnica do ensaio imunoenzimático (ELISA) sendo os os testes de biologia molecular no CEPEC (Com a orientação do Laboratório de Gastroenterologia e Hepatologia Tropical da Faculdade de Medicina da USP LGHT-FM/USP). O DNA e RNA viral serão extraídos de soro ou plasma . Os dados serão analisados utilizando o programa EPI-INFO (2000) do CDC de

Endereço: Rua Barão de Itapary nº 227
Bairro: CENTRO **CEP:** 65.020-070
UF: MA **Município:** SAO LUIS
Telefone: (98)2109-1250 **Fax:** (98)2109-1223 **E-mail:** cep@huufma.br

Atlanta /EUA e Excel (Windows 2007). A estimativa dos alelos será feitos a partir do método de contagem e também será utilizado o programa ADMIX.

Objetivo da Pesquisa:

Objetivo Primário:

Conhecer a prevalência das hepatites virais B, C e D e caracterizar os genótipos e subgenótipos desses vírus nos municípios de Urbano Santos, Humberto de Campos e de municípios da região do Baixo Munim (Axixá, Morros e Icatú)-MA e realizar estudos imunogenéticos e de ancestralidade nessa população.

Objetivo Secundário:

Identificar os indivíduos com sorologia positiva para os marcadores HBsAg, anti-HBc e/ou anti-HBs e anti-HCV. Identificar os indivíduos que apresentam o marcador sorológico para a hepatite D (anti-HDV) entre os portadores do HBsAg. Caracterizar os genótipos, subgenótipos (HBV) e subtipos (HCV) dos vírus da hepatite B, C e D através da técnica de sequenciamento direto. Estimar a ancestralidade genômica nessa população através de marcadores moleculares. Verificar a diversidade genética do complexo maior de histocompatibilidade (MHC) classe I e II, genes KIR e IL28B.Verificar a existência de associação entre maior percentagem de marcadores africanos e portadores das hepatites comparadas com não portadores. Verificar a existência de associação entre maior percentagem de marcadores africanos e genótipos virais. Verificar a existência de associação entre os alelos de MHC, KIR e IL28B com a presença da infecção viral pelos HBV e HCV.

Avaliação dos Riscos e Benefícios:

Riscos:

Desconforto na coleta de sangue, assim como a possibilidade de formação de edema na área da pulsão venosa.

Benefícios:Os pesquisadores destacam que os benefícios estão vinculados às ações de saúde de prevenção, diagnóstico precoce e tratamento além de informações precisas sobre as reais prevalências destas infecções trazendo impacto social de grande relevância.

Comentários e Considerações sobre a Pesquisa:

Protocolo com temática com relevante com proposta de diagnóstico precoce e conhecimento da prevalência das hepatites na região estudada trazendo impacto social de grande relevância.Estudo aprovado anterior à plataforma Brasil e inserido com emenda solicitando inclusão de novos participantes, prorrogação de execução e substituição da palavra "prevalência para Estudo" do título do estudo, sendo justificado que a alteração não reflete na metodologia. A inclusão de novos pesquisadores e prorrogação do tempo esta associada ao número da amostra(4.000 participantes.)

Considerações sobre os Termos de apresentação obrigatória:

O protocolo cumpre com as exigências em relação aos "Termos de Apresentação Obrigatória": Folha de rosto, Projeto de pesquisa, Termo de Consentimento Livre e Esclarecido (TCLE) orçamento e currículo do(s) pesquisador(es). Apresenta justificativa para solicitação de emenda.Atende, portanto às exigências da Resolução CNS/MS nº 466/12.

Recomendações:

Não há.

Conclusões ou Pendências e Lista de Inadequações:

Protocolo aprovado antes da plataforma Brasil com inserção de emenda com solicitações referente à prorrogação do tempo de coleta, inclusão de novos pesquisadores e modificações no título original. Documentos apresentados para justificativa da solicitação foram analisados sendo a emenda considerada aprovada.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

PROTOCOLO APROVADO por atender aos requisitos fundamentais da Resolução CNS/MS nº 466/12). Eventuais modificações ou emendas ao protocolo devem ser inseridas à plataforma encaminhada ao CEP-HUUFMA de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. Relatórios parcial e final devem ser apresentados ao CEP, inicialmente após a coleta de dados e ao término do estudo.

APÊNDICE B- Termo de consentimento livre e esclarecido

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

As hepatites virais são doenças que afetam o fígado e que possuem grande importância na saúde pública. Estas doenças na maioria das vezes não têm sintomas e quando apresentam podem ser fatais. Por esta razão convidamos o Senhor (a), a participar da pesquisa "**Estudo das Hepatites**
B, C e D nos municípios de Urbano Santos, Humberto de Campos e da Região do Baixo Munim, Maranhão, Brasil," a ser realizada, nos municípios de Urbano Santos, Axixá, Morros, Icatu e Humberto de Campos, coordenada pela Professora Doutora Adalgisa de Sousa Paiva Ferreira, residente à rua, Mitra, Ed. Maison Lafite Ap 1101. Renascença II, São Luís – MA. Telefone: (98) 3227-39.08; Fax-(98)32270131; e-mail adalgisa@terra.com.br. Este projeto tem como objetivo verificar o número de pessoas contaminadas com os vírus das Hepatites B (HBV), C (HCV) e D (HDV) na população dos municípios já citados. Também serão feitos exames de sangue para mostrar se há uma maior facilidade ou dificuldade para o aparecimento e desenvolvimentos dessas hepatites, de onde ela vem, (da África, Europa ou América) e se algum desses vírus tem alguma relação com as raças (branca, negra, indígena). Serão coletadas amostras de 15 mL de sangue. A coleta de sangue poderá causar um leve desconforto local, ou mesmo sensibilização e escurecimento do local de coleta, porém se o senhor (a) pressionar com algodão à parte puncionada, mantendo o braço estendido, sem dobrá-lo, a possibilidade de ficar roxo ou dolorido é muito pequena.

O material utilizado para coleta de sangue será descartado em recipientes apropriados para materiais pérfuro-cortante e todos os tubos de sangue serão mantidos em caixas térmicas com gelo. Todas as amostras de sangue serão utilizadas exclusivamente para este projeto. Ao término desta pesquisa o restante do material será descartado.

Em caso de dúvidas e/ou questionamentos relacionados com a ética da pesquisa, entrar em contato com o Comitê de Ética em Pesquisa do Hospital da Universidade Federal do Maranhão-HUUFMA, situado na Rua Barão de Itapary, 227, Centro, CEP 65020-070, São Luis-MA, telefone: (98) 2109 1250, email: cep@huufma.br. Quaisquer outras dúvidas a respeito da pesquisa, entrar em contato com a Prof. Dra. Adalgisa de Sousa Paiva Ferreira pelo telefone (98) 2109 1294.

Para realizar esta pesquisa, contamos com sua colaboração no sentido de responder as perguntas do formulário. Informamos que a pesquisa não lhe trará nenhum prejuízo, não afetará em nada se você tiver fazendo algum tratamento. Não haverá nenhum gasto com sua participação como também não receberá nenhum pagamento. Os exames serão totalmente gratuitos. Será garantido sigilo sobre as informações pessoais, tais como nome e RG. Sua participação não é obrigatória, mas é importante e, a qualquer momento poderá desistir de participar e retirar seu consentimento, como também poderá se recusar a responder quaisquer das questões que lhes causar constrangimento. Sua recusa não trará nenhum prejuízo em sua relação com os pesquisadores ou com a instituição.

Caso o exame seja positivo para algum dos vírus das hepatites, entraremos em contato para devida orientação. O resultado será de fundamental importância para melhorar o atendimento à população acometida da Hepatite B, C e D como também será usado para fins científicos e será publicado para uso da comunidade acadêmico-científica. Se concordar em participar, favor assinar as duas vias desse documento no final página sendo uma sua e outra do pesquisador. Agradecemos pela sua participação e nos colocamos à sua disposição para quaisquer esclarecimentos.

NOME E ASSINATURA DO SUJEITO OU RESPONSÁVEL: São Luís (MA)_____/_____/_____

_____ _____

(Nome por extenso) **(Assinatura)**

Assinatura do Pesquisador (a)_____

ANEXO B- NORMA INTERNA 01/2018

Regulamenta os artigos 27 e 28 do Regimento Interno do Programa de Pós-graduação em Ciências da Saúde, que trata da dissertação de Mestrado ou tese de Doutorado.

Considerando o contido na Resolução 1020-CONSEPE/2013;

Considerando a reunião do Colegiado do Programa, em _06_/_12_/_2017_;

O Colegiado do Programa de Pós-Graduação em Ciências da Saúde define os seguintes critérios de apresentação de Dissertação de Mestrado ou Tese de Doutorado:

Art. 1º. A dissertação de mestrado deverá obedecer a uma das seguintes alternativas:

I - **Formato tradicional.** Neste caso, a tese deverá conter: Elementos pré-textuais, Introdução, Referencial Teórico, Objetivos, Material e Métodos, Resultados, Discussão, Conclusões e Referencial teórico.

II - **Formato de artigo.** Neste caso, a dissertação deverá conter os elementos pré-textuais, introdução, referencial teórico, objetivos, material e métodos (opcional) e resultados (os quais serão divididos em capítulos, sendo um artigo por capítulo), considerações finais, conclusões e referências bibliográficas. Neste caso, o aluno deverá apresentar, nos resultados, pelo menos um artigo no qual deverá ser primeiro autor e redigido na forma exigida pela revista científica na qual se pretende publicá-lo.

III - Para a defesa, o aluno deverá anexar, à dissertação, o comprovante de submissão de um artigo em revista B2 ou superior, no qual o aluno seja autor, não necessariamente, primeiro autor.

Art. 2º. A tese de doutorado deverá obedecer a uma das seguintes alternativas:

I - **Formato tradicional.** Neste caso, a tese deverá conter: Elementos pré-textuais, Introdução, Referencial Teórico, Objetivos, Material e Métodos, Resultados, Discussão, Conclusões e Referencial teórico.

II - **Formato de artigo.** Neste caso, a tese deverá conter: Elementos pré-textuais, Introdução, Referencial Teórico, Objetivos, Material e Métodos (opcional), Resultados, Considerações finais, Conclusões e Referências bibliográficas. Serão aceitos como resultados da tese os artigos nas seguintes configurações:

a) Um (1) artigo científico completo (*full paper*) e um (1) artigo de revisão, redigidos na forma exigida pelas revistas científicas em que se pretende publicá-los;
b) Dois (2) ou mais artigos científicos completos (*full papers*), redigidos na forma exigida pelas revistas científicas em que se pretende publicá-los.

III – Para a defesa, o aluno deverá ter publicado, no curso do doutorado, no mínimo um artigo, não necessariamente como primeiro autor. **Entretanto,**

vale ressaltar que, os dados a serem apresentados pelo aluno devem ser os dados originais gerados por ele durante seu doutorado, os quais devem ter sido submetidos à publicação antes da defesa.

Art. 3º. **Somente são aceitáveis revistas científicas nacionais ou estrangeiras que atendam aos critérios vigentes estabelecidos pela CAPES, sendo no mínimo qualis B2 na área de Medicina I.**

Art. 4º. Os artigos científicos a serem apresentados no corpo da tese poderão estar redigidos em português ou inglês.

Art. 5º. A temática do artigo de revisão a ser incluído na tese de doutorado não poderá afastar-se daquela do tema central do artigo científico que o acompanha na composição da tese.

Art. 6º. As dissertações e teses deverão obedecer ao padrão que consta no anexo a esta resolução

Art. 7º. O mestrando somente terá direito ao Certificado quando apresentar ao Colegiado as cópias da versão definitiva da Dissertação, bem como uma versão eletrônica, em arquivo único salvo em PDF.

Art. 8º. O doutorando somente terá direito ao Certificado quando apresentar ao Colegiado as cópias da versão definitiva da Tese, uma versão eletrônica, em arquivo único salvo em PDF, bem como, o comprovante de aceite pela revista cientifica de pelo menos um dos artigos relacionados com sua tese.

Art. 12º. Estas Normas entram em vigor a partir da data da aprovação no Colegiado.

São Luís, ____/____/____.

_____

Prof. Dra. Flávia Raquel Fernandes do Nascimento

Coordenadora