UNIVERSIDADE DE PASSO FUNDO
FACULDADE DE AGRONOMIA E MEDICINA VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOEXPERIMENTAÇÃO

DESENVOLVIMENTO E CARACTERIZAÇÃO DA PROTEÍNA RECOMBINANTE ORF2 DO VÍRUS DA HEPATITE E GENÓTIPO 3

DISSERTAÇÃO DE MESTRADO

Denise Ramos de Almeida

Passo Fundo, RS, Brasil
2015

DESENVOLVIMENTO E CARACTERIZAÇÃO DA PROTEÍNA RECOMBINANTE ORF2 DO VÍRUS DA HEPATITE E GENÓTIPO 3

Denise Ramos de Almeida

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Bioexperimentação, Área de Concentração em Bioexperimentação, da Faculdade de Agronomia e Medicina Veterinária da Universidade de Passo Fundo (UPF), como requisito parcial para a obtenção do grau de **Mestra em Bioexperimentação.**

Orientador: Prof. Dr. Rafael Frandoloso

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Elaborada por
Denise Ramos de Almeida

Como requisito parcial para a obtenção do grau de
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Comissão Examinadora

Rafael Frandoloso, Ph.D., UPF
(Orientador/Presidente)

Luiz Carlos Kreutz, Ph.D., UPF

DenizAnziliero, Dr. IMED

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“A coisa mais indispensável é reconhecer o uso que se deve fazer do próprio conhecimento.”

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LISTA DE ABREVIATURAS

ABNT	Associação Brasileira de Normas Técnicas
ALT	Amino Alanino Transferase
BNDE	Banco Nacional de Desenvolvimento Econômico
cDNA	Cópia do DNA complementar
DNA	Ácido desoxiribonucleico
ELISA	Ensaio Imunoenzimático
HEV	Vírus da Hepatite E
Gt	Genótipo
Kb	Kilobases
Kg	Kilograma
mg	Miligramas
µg	Micrograma
µl	Microlitro
ORF	Janela Aberta de Leitura
PCR	Reação em Cadeia da Polimerase
RNA	Ácido Ribonucleico
RT PCR	Reação em cadeia da Polimerase via Transcriptase Reversa
SDS	Duodecil Sulfato de Sódio
PAGE	Eletroforese em Gel de Poliacrilamida
UPF	Universidade de Passo Fundo

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Bioexperimentação
Universidade de Passo Fundo

DESENVOLVIMENTO E CARACTERIZAÇÃO DA PROTEÍNA RECOMBINANTE ORF2 DO VÍRUS DA HEPATITE E GENÓTIPO 3

Autor: Denise Ramos de Almeida
Orientador: Dr. Rafael Frandoloso
Passo Fundo, 2015

Hepatite E é uma doença infectocontagiosa viral causada pelo vírus da hepatite E (HEV), a qual possui destacada importância em saúde pública. Desde o seu descobrimento há 30 anos, surtos epidêmicos são associados com o consumo de águas contaminadas com o HEV, principalmente naqueles países onde o saneamento básico é deficitário ou mesmo inexistente. Por outro lado, países industrializados também notificam esta infecção e associam a mesma com o consumo de alimentos infectados com o HEV ou com viajantes que retornam de zonas endêmicas. Neste trabalho, descrevemos o desenvolvimento e a caracterização de um antígeno recombinante derivado da proteína ORF2 do HEV genótipo 3 (gt3). O RNA viral foi obtido a partir de fezes procedentes de suínos naturalmente infectados com o HEV gt3. A sequência codificante de 267 aminoácidos localizados no extremo carboxilo terminal da proteína ORF2$^{(394\text{-}661)}$ foi clonada dentro do vetor pET20a e expressada em *Escherichia coli* cepa ER2566. A sequência de aminoácidos do fragmento proteico clonado foi alinhada com diferentes sequências da proteína ORF2 do HEV gt3 isolado de humanos e de suínos, revelando uma homologia de 98%. A expressão da proteína recombinante ORF2p foi obtida mediante a indução com 0.4mM de IPTG e sua purificação, obtida mediante cromatografia líquida de proteína. O método de expressão utilizado para produzir a ORF2p foi altamente eficiente, rendendo 10 mg de proteína solúvel por litro de cultivo de *E. coli*. As propriedades imunogênicas da ORF2p foram avaliadas em Ratas Wistar. Em paralelo, suínos HEV negativos foram experimentalmente infectados com o HEV gt3. A proteína ORF2p demonstrou-se altamente imunogênica em ratas, rendendo altos títulos de anticorpos capazes de reconhecer o antígeno recombinante homólogo bem como a ORF2 do HEV gt3 presente nas fezes dos animais experimentalmente infectados. De forma inversa, demonstramos a capacidade dos anticorpos anti-HEV procedentes de macacos *Cynomologus* de reconhecer especificamente a proteína recombinante ORF2p, destacando a antigenicidade do antígeno desenvolvido. Nenhum dos leitões infectados apresentou sinais clínicos compatíveis com a doença durante o experimento ou, alterações macroscópicas durante a necropsia. Apesar do quadro clínico normal, HEV foi detectado nas fezes de 3 dos 4 leitões 14 dias após a infecção por PCR e, uma semana depois, mediante dot blot. Em conclusão, este estudo apresenta o desenvolvimento de um fragmento imunogênico da proteína ORF2 do HEV gt3, com excelentes características antigênicas, as quais convertem a ORF2p, em um candidato potencial para o desenvolvimento de ferramentas de diagnóstico para a detecção do HEV gt3 no Brasil como também, para o desenho de vacinas contra este genótipo em específico

ABSTRACT

Dissertação de Mestrado
Programa de Pós-GraduaçãoemBioexperimentação
Universidade de Passo Fundo

Development and characterization of a recombinant ORF2 protein based on C-terminal sequence of the Hepatitis E Virus genotype 3 isolated in Brazil

Author: Denise Ramos de Almeida
Teacher: Dr. Rafael Frandoloso
Passo Fundo, 2015

Hepatitis E, caused by hepatitis E virus (HEV), is a viral infectious pathology of great importance in the public health. Since HEV discovery 30 years ago, many cases of waterborne epidemics have been attributed to this virus. These outbreaks were registered in developing countries with poor or no sanitation, where drinking water was contaminated with infected fecal material. Hepatitis E is also reported in many industrialized countries probably due to consumption of HEV-positive swine meat or to travelers returning from endemic regions. In this study, we present the development and characterization of a recombinant antigen from ORF2 HEV gt3. Viral RNA was isolated from swine feces infected with the native virus. 267 residues from the C-terminal ORF2$^{(394\text{-}661)}$ coding sequence were cloned into the pET20a vector and expressed in *Escherichia coli* ER2566. The amino acid sequence cloned was aligned against other human or swine HEV gt3 ORF2 sequences, revealing a 98% homology. The expression of ORF2p recombinant protein was achieved with 0.4mM of IPTG and purified by protein liquid chromatography. The method of expression used to produce ORF2p was highly efficient, yielding 10mg of soluble protein per liter of *E. coli* medium. Wistar rats were inoculated with ORF2p to test its immunogenic properties. In parallel, HEV-negative swine were experimentally infected with HEV gt3. The study of ORF2p immunogenic capacity in rats resulted in a high antibody titration able to recognize both the homologous antigen and the native HEV gt3 ORF2 present in infected stool. Furthermore, anti-HEV antibodies from *Cynomolgus* monkey recognized ORF2p recombinant protein, underlying its antigenicity. None of the infected piglets showed clinical signs compatible with the disease during the experiment or macroscopic alterations during the necropsy. Despite the overall healthy clinical picture, HEV was detected by PCR 14 days post-infection in 3/4 piglets' feces and one week later by dot blot. In conclusion, this study proved the immunogenic and antigenic properties of the recombinant protein ORF2p. According to our results, ORF2p is a valid candidate for the development of diagnostic tools to detect HEV gt3 circulating in Brazil as well as for the design of a vaccine targeting this specific genotype.

1. INTRODUÇÃO

O vírus da hepatite viral E, é um vírus RNA, não envelopado, icosaédrico, com 27-34 nm de diâmetro e pertencente à família *Heperiviridae*(1). Seu genoma possui três janelas abertas de leitura (ORFs), cujas sequências nucleotídicas codificam proteínas não estruturais (ORF1) e duas proteínas (ORF2 e ORF3) envolvidas na formação do capsídeo e na interação com o citoesqueleto celular, respectivamente (2,3).

O HEV pode ser classificado em quatro genótipos principais (gt 1 - 4) (1). Os gt1 e 2 são encontrados exclusivamente em humanos, e constituem a causa primária de hepatite E em regiões onde o saneamento básico é precário ou inexistente (4). Por outro lado, infecções produzidas pelos gt3 e 4 tem sido demonstradas principalmente em países desenvolvidos e relacionadas com o consumo de carne procedente de suínos infectados (5), destacando o potencial zoonótico (6).

O diagnóstico da hepatite E é realizado principalmente mediante sorologia com a detecção de IgM e IgG (10, 13, 14), pois o curto período de viremia, limita a detecção viral através de técnicas moleculares (15).

No Brasil, estudos realizados em humanos e suínos revelaram a presença e a circulação do HEV gt3(6, 16). No entanto, faltam dados soro-epidemiológicos robustos que demonstrem, de forma macro, o cenário desta infecção. Atualmente os kits comerciais utilizados para o diagnóstico da hepatite E incluem poliproteínas recombinantes derivadas das proteínas ORF2 e OFR3 dos genótipos 1 e 2. A capacidade destes kits de detectar anticorpos anti-HEV gt3 e 4 é limitada (17), ressaltando a necessidade de uma atualização para outros genótipos.

Do ponto de vista clínico, a infecção é assintomática em suínos (7) sendo que em humanos há potencial em desenvolver hepatite aguda (8-10), especialmente grave em pacientes imunocomprometidos, com doenças hepáticas pré-existentes, idosos e gestantes (11, 12).

Neste estudo, levando em consideração o importante rebanho suíno existente no Brasil, os escassos dados soroepidemiológicos de hepatite E e a inexistência de um kit comercial baseado no principal genótipo detectado no Brasil, descrevemos o desenvolvimento e a caracterização de um antígeno recombinante baseado na estrutura da proteína ORF2 do HEV gt3, com potencial uso diagnóstico e imunopreventivo para hepatite. Os resultados dos experimentos estão descritos na forma de um artigo científico intitulado "**Development and characterization of a Brazilian candidate antigen for Hepatitis E Virus genotype 3 diagnosis"**, o qual foi submetido para publicação no periódico FEMS Microbiology Letters.

2. REVISÃO DA LITERATURA

2.1 O vírus da Hepatite Viral E

Descoberto em 1983, o vírus da Hepatite E é um pequeno vírus icosaédrico (27-34 nm), não envelopado, RNA de fita simples positivo e pertencente à família *Hepeviridae* (8, 18).

A doença foi descrita em 1980, durante uma avaliação retrospectiva de duas epidemias que ocorreram na Índia na década de 50 (1955 e 1956). Na época, não foram encontrados marcadores sorológicos compatíveis com as hepatites A ou B (7), e então, o agente epidêmico foi nomeado como vírus da Hepatite E, sendo mais tarde caracterizado molecularmente (8).

2.1.1 Genoma

O genoma do Vírus da Hepatite Viral E tem aproximadamente 7,5 kilobases (kb), contendo no final nos extremos 5' e 3' duas regiões curtas não codificantes (UTRs) de 26 e 68 nucleotídeos, respectivamente (19). Entre estas duas UTRs, se apresentam três janelas abertas de leitura (ORF's) parcialmente sobrepostos, chamados de ORF1, ORF2 e ORF3. ORF1 têm início no extremo 5' e se estende por aproximadamente 5 Kb dentro do genoma. ORF2 inicia na posição nucleotídica 39 do extremo - 3', e a ORF3, sobrepõe parcialmente ambas sequências ORF1 e ORF2, tendo um comprimento de somente 369 pb (20).

2.1.1.1 ORF 1

A ORF1 codifica um conjunto de proteínas com funções não estruturais. Está constituída por diferentes elementos funcionais como a RNA polimerase dependente de RNA (RdRp), RNA Helicase (Hel), Protease papaína (PC) e Metiltransferase (MeT). Estas proteínas estão envolvidas na replicação do material genético viral (enzima RdRp), na transcrição a partir do cDNA (Metiltransferase), e na criação das formas maturas e ativadas das proteínas virais (Protease papaína).

2.1.1.2 ORF2

A segunda janela aberta de leitura (ORF2) codifica a proteína do capsídeo, constituída por 660 aminoácidos (aa). Esta proteína é translocada através do retículo endoplasmático (RE) durante a translação, através de uma sequência hidrofóbica localizada no N-terminal da proteína, a qual é necessária para sua localização final na superfície celular (21).

A proteína ORF2 é N-glicosilada nos aminoácidos 137, 310 e 562, sem a qual o vírus não é infeccioso. As proteínas ORF2 não glicosiladas são retro-translocadas para o citoplasma,

permanecendo estáveis e indicando que a proteína viral mimetiza um substrato do proteossomo para ter acesso ao citoplasma (9, 19).

A sobrexpressão da proteína ORF2 em baculovírus resulta na produção de grande quantidade de ORF2, chamadas de partículas virais associadas (VLP) com destacado potencial imunogênico e antigênico (22-24)

2.1.1.3 ORF 3

A terceira janela aberta de leitura codifica uma proteína de 123 aminoácidos. A exata função desta proteína não foi ainda bem determinada, entretanto, dados contraditórios descrevem sua interação com muitas proteínas do hospedeiro e da proteína do capsídeo viral (1, 10).

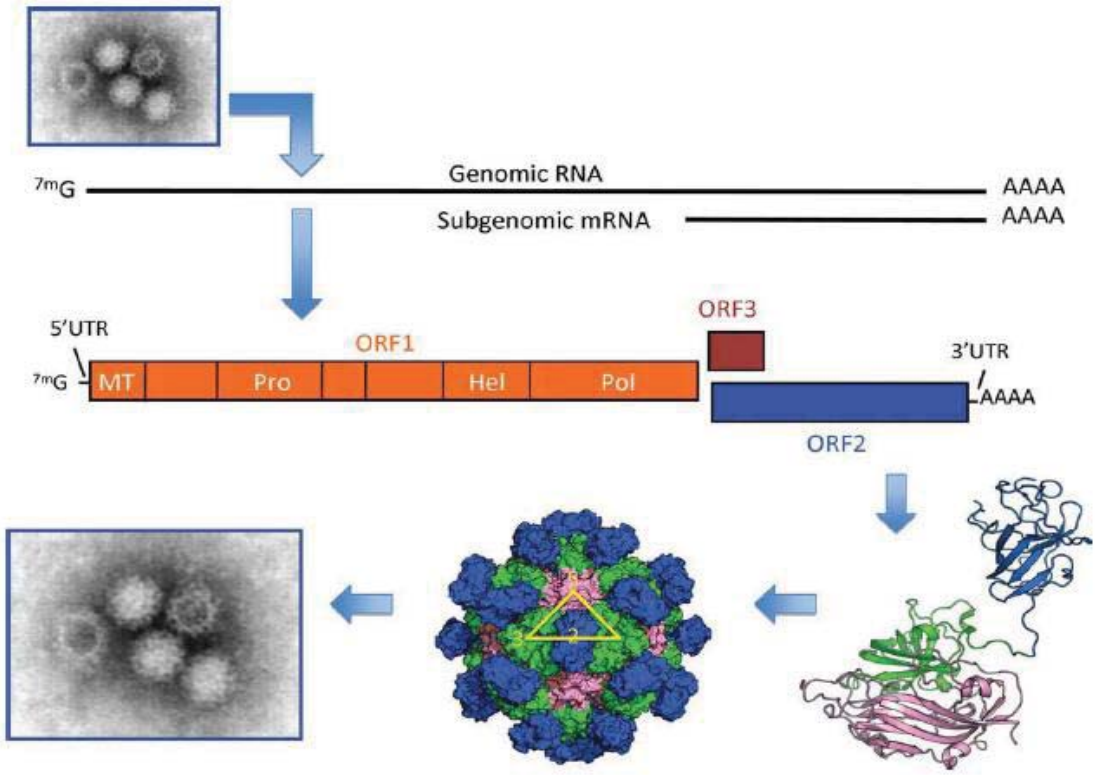

FIGURA 1. HEV e seu genoma. Representação esquemática do RNA genômico contendo 7,2 Kb e duas regiões UTR. Durante replicação do genoma, um subgenoma de RNA de aproximadamente 2 Kb também é produzido. Formado por três ORFs que codificam proteínas não estruturais (ORF1, laranja), a proteína do capsídeo (ORF2, azul) e uma proteína reguladora (ORF3, marrom). A ORF2 monomérica possui três domínios (representados em rosa, verde e azul) que resultam em diferentes elementos estruturais sobre a superfície do virion (25).

2.1.2 Genótipos

Baseado na variabilidade genômica, os HEV isolados, podem ser classificados em quatro genótipos: Genótipo 1, vem sendo identificado em episódios epidêmicos em países em desenvolvimento na Ásia e na África; Genótipo 2, foi descrito no México e na África; Genótipo 3, tem distribuição ampla e foi isolado em casos esporádicos de Hepatite viral E aguda e/ou de suínos domésticos em 22 países (Argentina, Austrália, Áustria, Cambodia, Canadá, França, Alemanha, Grécia, Itália, Japão, Corea, México, Holanda, Nova Zelândia, Rússia, África do Sul, Espanha, Taiwan, Tailândia, Reino Unido e estados Unidos); e o Genótipo 4, descrito em humanos e suínos domésticos na China, Índia, Indonésia, Japão, África do Sul, Taiwan e Vietnam (Fig. 2 e 3) (1, 8, 16, 26, 27)..

No Brasil estudos de soroprevalência demonstraram a presença de anticorpos anti-HEV em diferentes grupos populacionais, fundamentalmente em mineiros e na Bacia Amazônica (6,1%) (28). Em São Paulo, pacientes submetidos à hemodiálise apresentaram prevalência de 4,9% de anticorpos anti-HEV (29). Em Salvador, Bahia, existe uma prevalência de 2% de hepatite viral E entre doadores de sangue (30).

No Laboratório de Referência Nacional para Hepatites Virais / Fiocruz / RJ (CRNHV), entre janeiro de 1994 e dezembro de 1996, foram diagnosticados 147 casos de hepatite viral aguda não A-C, com prevalência de anticorpos anti-HEV de 2,1% (31). No Rio de Janeiro, por sua vez, foi observado uma prevalência de 2,4% na comunidade de Manguinhos (31).

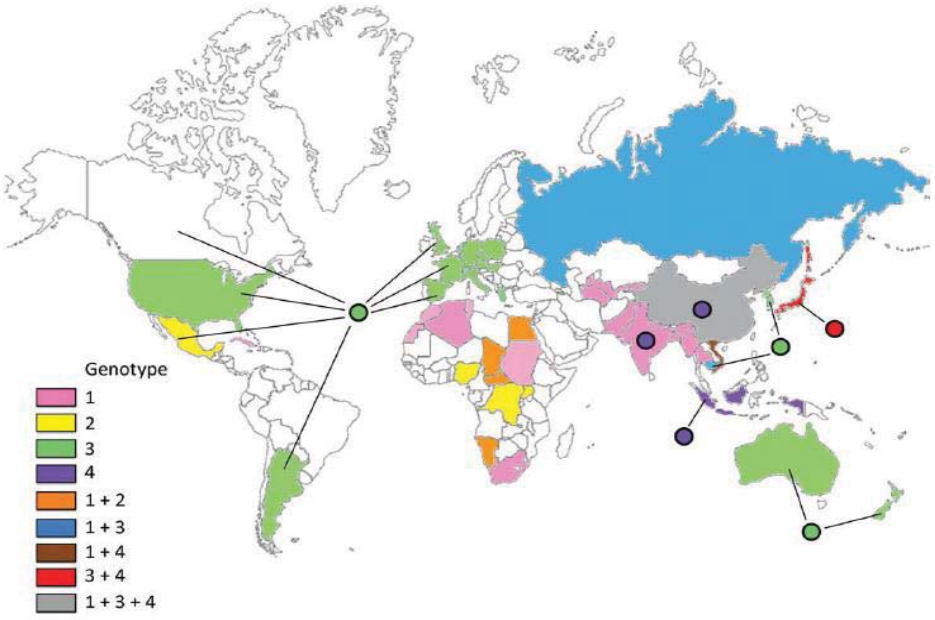

FIGURA 2. Distribuição dos genótipos do HEV isolados de humanos e animais (predominantemente suínos). As cores atribuída a um país e o círculo associado a ele, representa o genótipo viral predominante em humanos e animais (25).

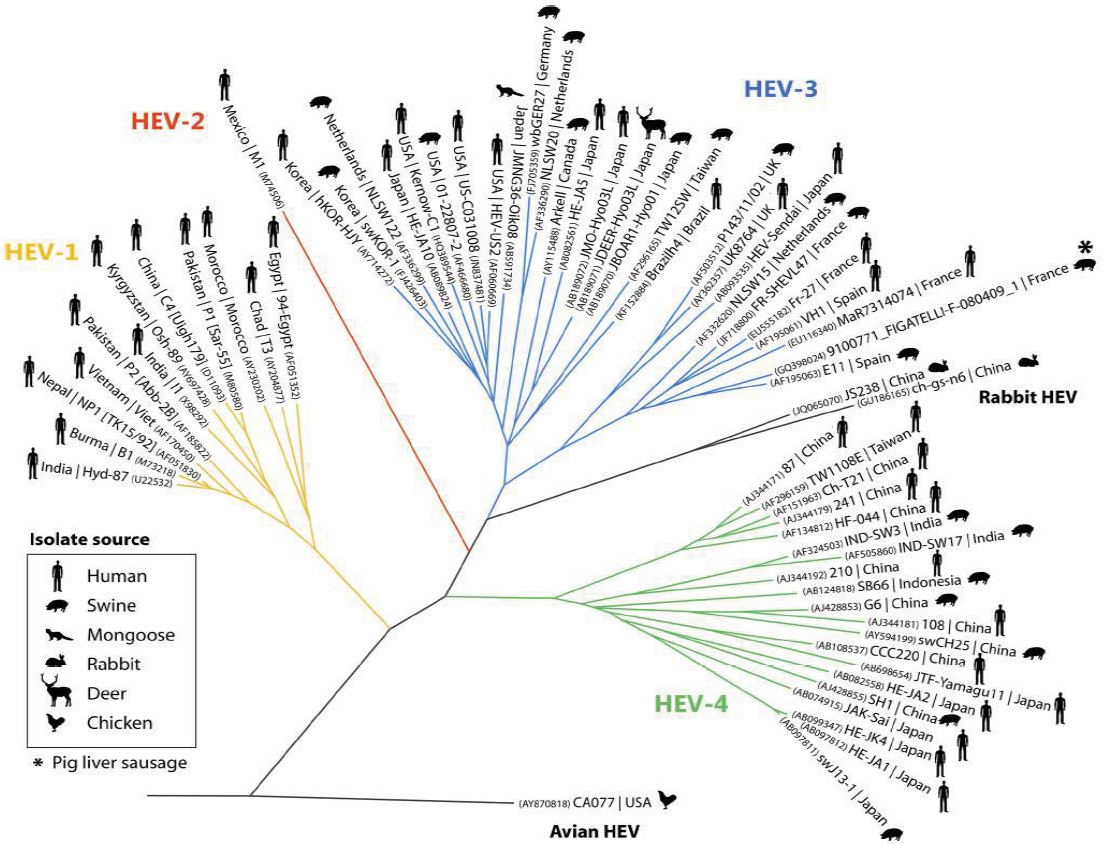

FIGURA 3. Árvore filogenética global do HEV baseada na sequência nucleotídica da proteína do capsídeo. Genótipo 1 e 2 (HEV-1 e HEV-2) circulam entre os humanos, primariamente na Ásia e África enquanto os genótipos 3 e 4 (HEV-3 e HEV-4), tem animais como reservatórios zoonóticos, e são comumente transmitidos através de alimentos (10).

2.1.3 Replicação viral

O ciclo de replicação do HEV parece ser similar ao de outros vírus RNA não envelopados. Entretanto, poucas pesquisas tem sido realizadas sobre o ciclo de replicação do HEV devido a falta de linhagens celulares eficientes para trabalhar-se com este patógeno *in vitro*. Com relação a interação do vírus com as células do hospedeiro, ainda permanece oculto quais receptores celulares estão envolvidos primariamente na união do HEV, no entanto, parece que resíduos de sulfato de heparina estão envolvidos neste processo (32).

2.1.4 Patogênese e Patologia

Muitos aspectos relacionados com a patogênese da infecção permanecem desconhecidos, devido entre outras razões, aos escassos estudos realizados com este microrganismo e a dificuldade de reproduzir a doença *in vitro* (1).

A via de infecção viral ocorre, principalmente, por via oral através do consumo de águas ou alimentos contaminados (17). O lugar de replicação primário do vírus ainda não foi identificado, no entanto, presume-se ser no trato intestinal (25). Da mesma maneira, não está

claro como o vírus chega aos hepatócitos, mas acredita-se que seja através da veia porta. Uma vez nos hepatócitos, o vírus se replica no citoplasma (20) e, consequentemente, ocorre a liberação de novas partículas virais na bile e no sangue (1).

Estudos realizado em voluntários (infecção oral), demonstraram a presença de viremia 22 dias após a exposição. Partículas virais foram identificadas em fezes, mediante microscopia eletrônica, 34 dias após a exposição e anticorpos anti-HEV foram detectados aos 41 dias de infecção, podendo ainda ser detectados após 48 meses da exposição (8).

As manifestações da infecção vão desde uma doença subclínica até o desenvolvimento de uma hepatite fulminante, e o período de incubação varia de 15 a 60 dias, com média de 40 dias (8, 13). A infecção do HEV tem um espectro amplo de manifestações clínicas, sendo a hepatite aguda sintomática a principal. Em pacientes com doenças hepáticas pré-existentes, idosos e gestantes, o curso torna-se grave, podendo o polimorfismo genético contribuir para a gravidade da doença (Fig. 4) (11, 12).

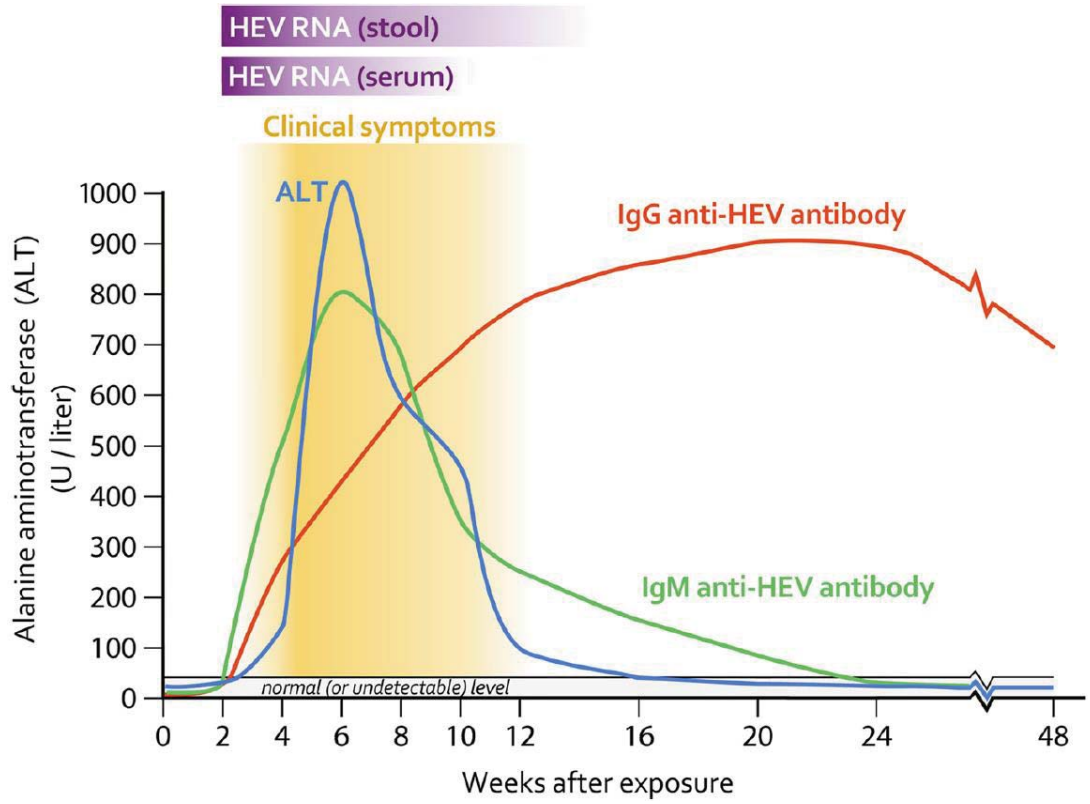

FIGURA 4. Curso da infecção aguda do HEV. A hepatite E aguda é caracterizada por sintomas como febre, anorexia, vômitos e icterícia que iniciam várias semanas após a exposição inicial. O inicio dos sintomas clínicos coincidem com o aumento do nível da enzima hepática alanino transaminase (ALT). Sintomas podem persistir por algumas semanas ou até um mês ou mais. O nível de ALT retorna ao normal durante a convalescência. O RNA viral pode ser detectado no sangue e nas fezes no início da infecção, no entanto, a viremia é de difícil detecção. Os títulos de IgM anti-HEV aumentam rapidamente e começam a decair semanas após a infecção,

coincidindo com o aumento dos títulos de IgG anti-HEV os quais podem ser detectados durante meses ou mesmo anos após a infecção (10).

2.2 Diagnóstico laboratorial

Infecções produzidas pelo vírus da Hepatite E não podem ser distinguidas de outros tipos de hepatites agudas somente com base nas características clínicas. Dessa maneira, o diagnóstico laboratorial torna-se imprescindível, e busca detectar anticorpos específicos contra o HEV ou a presença de RNA viral em pacientes infectados (13, 33).

Basicamente dois métodos de diagnóstico são utilizados para o diagnóstico de HEV: O primeiro método consiste em um teste sorológico do tipo ELISA. Este teste utiliza, como antígeno, poliproteínas recombinantes derivadas da proteína ORF2, e tem por objetivo detectar a presença de anticorpos específicos (IgG e IgM) no soro de pacientes contra o HEV. Também, pode ser utilizado para determinar se um indivíduo esteve em contato com o vírus de forma aguda ou crônica, já que as imunoglobulinas do tipo IgG contra o HEV permanecem circulando em títulos detectáveis durante muitos anos (2 – 13 anos) (34).

O segundo método consiste na detecção molecular de ácidos nucleicos e pode ser utilizado para detectar o RNA viral no soro, bile e nas fezes. No entanto, este teste somente é efetivo para detectar uma infecção ativa, enquanto a detecção sorológica de IgM fornece uma informação de infecção aguda ou recente (8) .

Os kits comerciais utilizados para o diagnóstico sorológico da hepatite viral E incluem, de forma restrita, fragmentos da OFR2 e OFR3 dos genótipos 1 e 2. Os genótipos 3 e 4 são os mais prevalentes nos países industrializados, e em vários estudos demonstram falhas na para detecção de anticorpos específicos contra o genótipo 3 (17) e mesmo, uma subnotificação dos casos autócones(17, 35).

2.3 Tratamento

A hepatite viral E pode ser autolimitada, e desta forma, muitos pacientes não precisam de tratamento específico. Outros, todavia, podem evoluir para falência hepática aguda ou subaguda e nesse caso, necessitam de tratamento intensivo para controlar a insuficiência hepática. Em casos mais graves, torna-se inevitável o transplante hepático (8, 25, 36).

Na gestação, há aumento do risco de coagulopatia e hemorragia pós-parto, sendo necessário uso de hemocomponentes para o controle de hemorragias (25) .

Terapia com interferon alpha-2α / alpha-2β ou ribavirina por 3 a 12 meses tem sido usado em pacientes com infecção por HEV crônica com sucesso (25).

2.4 Prevenção

A Hepatite Viral E pode ser prevenida com o uso de água tratada, depósito adequado das fezes e medidas de higiene pessoal. Uso de cloro na água é útil. Medidas sanitárias envolvendo alimentos a base de carne de suínos devem ser implantadas nas áreas de transmissão zoonóticas, orientando a população a realizar o cozimento adequado desses produtos (25).

2.5 Vacina

Algumas vacinas contra hepatite viral E estão em fase clínica de pesquisa, fundamentalmente na China, onde a presença do HEV é endêmica. As vacinas atualmente estudadas são compostas por proteínas recombinantes derivadas da ORF2 (proteína do capsídeo) e apresentam excelentes resultados de soro-conversão e proteção em humanos (25, 37).

3. CAPÍTULO 1

Development and characterization of a Brazilian candidate antigen for Hepatitis E Virus genotype 3 diagnosis

Denise Ramos de Almeida[1], Michela Miani[1], Rafael Pandolfi[1], Luis Tondo[1], Maikel Luis Colli, Fernando Rosado Spilki[2], Noemi Rovaris Gardinali[3], Marcelo Alves Pinto[3], Luiz Carlos Kreutz[1], Rafael Frandoloso[1*]

Affiliation: 1- Laboratório de Imunologia e Microbiologia Avançada, Programa de Pós-Graduação em Bioexperimentação, Universidade de Passo Fundo (UPF), Passo Fundo, RS, Brazil; 2- Laboratório de Microbiologia Molecular (LMM), Instituto de Ciências da Saúde (ICS), Universidade Feevale, Novo Hamburgo, RS, Brazil; 3- Laboratório de Desenvolvimento Tecnológico em Virologia/IOC – Fiocruz, RJ, Brazil.

*Corresponding author. Rafael Frandoloso, Faculdade de Agronomia e Medicina Veterinária, Universidade de Passo Fundo, Campus I, Bairro São José. CEP 99052-100. Telephone: +55 54 3316 8485; Fax: +55 54 3316 8163. E-mail: rfran@upf.br

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Abstract

Hepatitis E, caused by hepatitis E virus (HEV), is a viral infectious pathology of great importance in the public health. Hepatitis E outbreaks were registered in developing countries with poor or no sanitation, where drinking water was contaminated with fecal material, but also in many industrialized countries probably due to consumption of HEV-positive swine meat. In this study, we present the development and characterization of a recombinant antigen from ORF2 HEV genotype 3. Viral RNA was extracted from swine feces infected with the native virus. 267 residues from the C-terminal ORF2$^{(394\text{-}661)}$ coding sequence were cloned into the pET20a vector and expressed in *Escherichia coli* ER2566. Recombinant protein was purified by liquid chromatography and the fragment obtained a 98% homology against other human or swine HEV genotype 3 ORF2 sequences. Wistar rats were inoculated with ORF2p, developing antibodies able to recognize both the homologous antigen and the native HEV genotype 3 ORF2 present in infected stool. In parallel, HEV-negative swine were experimentally challenged with HEV genotype 3. ORF2 was detected by PCR 14 days post-inoculation in 3/4 piglets' feces and one week later by dot blot. In conclusion, this study proved the immunogenic and antigenic properties of the recombinant protein ORF2p.

Introduction

Hepatitis E is endemic in developing countries from Asia, Africa and South America (Arankalle *et al.* 1995; Chandra *et al.* 2008). Infection generally occurs by drinking water contaminated with hepatitis E virus, indicating a preferential fecal-oral route of transmission (Krawczynski 1993). Virus contaminated food (Meng 2011) or blood transfusion (Khuroo, Kamili and Yattoo 2004) might also be indicated as potential source of infection

Hepatitis E virus (HEV) is a small, non-enveloped, plus-stranded RNA virus (Reyes *et al.* 1990). The HEV genome contains three open-reading frames (ORFs): ORF1 encodes for a protein containing domains present in nonstructural proteins (Koonin *et al.* 1992), ORF2 codifies for the viral capsid protein (Li *et al.* 1997) and ORF3 codifies for a small cytoskeleton-associated phosphoprotein (Zafrullah *et al.* 1997).

There are four known genotypes (genotype1 to 4), all of them have been detected in human. While HEV genotype1 and HEV genotype 2 have been detected exclusively in humans, HEV genotype 3 and HEV genotype 4 have also been found in several animal species (Krain, Nelson and Labrique 2014).

Epidemiologically, HEV genotype 1 and 2 cause outbreaks of hepatitis E in Asia, Africa, Central and South America (Escriba *et al.* 2008; Garcia *et al.* 2012; Teo 2012; Villalba Mde *et al.* 2008). HEV genotype 3 and 4 are responsible for sporadic cases of hepatitis E worldwide and their presence has been documented in domestic pigs from several industrialized countries. The nucleotide sequence similarity between swine and human HEV putatively allows the cross-species infection (Meng *et al.* 1998; Meng *et al.* 1997; Yazaki *et al.* 2003; Zhang *et al.* 2010).

71 Industrialized countries with intensive swine industry (such as Spain, France,
72 Germany, Japan and China) and pork meat consumption alerted the population about
73 the autochthonous circulation of HEV genotype 3. In these studies, they identified this
74 genotype as the agent causing several cases of acute hepatitis E in human (Legrand-
75 Abravanel *et al.* 2010; Lens *et al.* 2015; Wichmann *et al.* 2008; Yazaki *et al.* 2003;
76 Zhang *et al.* 2010).
77 Diagnosis of HEV infection is mainly dependent on serology as the viremia is
78 limited to the acute phase of infection in human beings (Jimenez de Oya *et al.* 2009).
79 Commercial kits to detect anti-HEV antibodies in human serum are designed on short
80 ORF2 and ORF3 fragments of HEV genotype 1 or HEV genotype 2 (Jimenez de Oya
81 *et al.* 2009). There is no available kit to specifically detect HEV genotype 3, which is
82 the most prevalent genotype in humans and swine in industrialized countries (Daniel *et*
83 *al.* 2004; Herremans *et al.* 2007). As a result, HEV genotype 3 infection could be under-
84 estimated in human and in swine population.
85 In this study we produced a recombinant ORF2 antigen from HEV genotype 3
86 isolated in Brazil. The immunogenic and antigenic properties of this antigen support it
87 to develop diagnostic assays or its use as a targeted vaccine against HEV genotype 3.
88

Materials and methods

Viral RNA isolation and cDNA synthesis

Viral RNA extraction was performed on stool of HEV genotype 3 naturally infected swine (LADTV-IOC/Fiocruz, Rio de Janeiro). Briefly, 5 g of feces were suspended in 5 ml RPMI 1640 (Invitrogen, CA) and centrifuged for 20 mins 13,000 ×*g* at 4°C. Supernatant was filtered (0.22 µm, *TPP*) and viral RNA was isolated with QIAmp Viral RNA Mini (Qiagen, The Nederlands) according to manufacturer instructions. Isolated RNA purity and concentration were determined by spectrophotometry (NanoPhotometre, Implen, Germany). Samples were stored at -80°C.

cDNA synthesis was performed by reverse transcription (RT) using QuantiTec Reverse Transcription (Qiagen) according to manufacturer recommendations.

Cloning and sequencing of ORF2 gene

A specific pair of primers (orf-2F and orf-2R) were designed to amplify a 801 nucleotides fragment from the HEV genotype 3 ORF2 $^{(394\text{-}661)}$ C-terminal (GenBank access n° AB362842.1). Fragment was amplified by PCR (final volume 50 µl) with 500 nM of each primer (sequence available upon request), 3 U of Pfu DNA polymerase, 5 µl 10x buffer supplemented with $MgSO_4$, 200 µM dNTPs (Promega, WI) and DNAse-free water (Sigma, Germany). PCR cycles were as follows: initial denaturation set at 95°C for 10 min followed by 35 cycles of 95°C for 60 sec, 55°C for 45 sec and 72°C for 90 sec. Fragment DNA 3'-end was adenylated and cloned inside a pGEM-T-Easy vector (Promega) according to manufacturer instructions. The vector was transformed in competent *Escherichia coli* JM109 strain (Promega). Positive clones

(pGEM-T-ORF2p) were confirmed by PCR and sequencing was performed using pUC/M13 primers (Promega). The sequence was aligned with several human or swine fragments from Spain (AFJ06417.1), France (AHC55357.1), Germany (AHC54588.1), Hungry (AEA4882.1), Japan (BAE98089.1), China (ACV66756.1), Thailand (ACB15470.1) and Brazil (EF491203.1). The alignment was done using Gene Inspector 2.0 software.

Subcloning and expression of ORF-2 protein

The pGEM-T-ORF2p vector was digested with BamHI and HindIII restriction enzymes (Promega) to obtain a DNA fragment containing the ORF2p that was separated on 1% agarose gel stained with GelRed (Unisen, Hong Kong), cut and purified with Wizard SV Gel (Promega). The fragment was inserted into a pET20 modified expression vector (kindly provided by Dr. Schryvers) previously digested with the same restriction enzymes. The pET20 vector express a fusion protein that has on its N-terminal 6 histidine residues followed by a maltose-binding protein (Mbp) and a restriction site for tobacco etch virus (TEV) protease, all preceding the ORF2 coding region. The resulting plasmid construct, pET20-His-Mbp-TEV-ORF2p, was then transformed in competent *E. coli* ER2566 strain (New England BioLabs® Inc.) and Mbp-TEV-ORF2p fusion protein expression was stimulated overnight with different isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) concentrations (0.1 – 0.5 mM). Culture medium was centrifuged for 1 hour at 8,000 ×*g* and bacterial pellet was resuspended in NTA buffer (20mM NaH_2PO_4, 500mM NaCl, 20mM Imidazole, pH8.0). Bacteria were lysed with 3 cycles of sonication and protein-enriched supernatant was purified in a nickel sepharose chromatography column (GE Healthcare), and eluted with NTA buffer containing 250 mM imidazole

(Sigma). Purified Mbp-ORF2p was exposed to TEV protease, dialyzed (10mM Hepes pH 6.0) and purified with a Q sepharose column (GE Healthcare). The highly pure ORF2p recombinant protein was obtained with 75 mM NaCl (Sigma).

Polyclonal serum production

Wistar rats (n=4) were immunized subcutaneously with 50µg of recombinant ORF2p (n=2) or Mbp-ORF2p (n=2) combined with Freund's adjuvant (Sigma) in a 1:1.2 ratio. The first immunization was performed with Freund's complete adjuvant while Freund's incomplete adjuvant was used for subsequent injections at 21 and 42 days after priming. Animal blood was collected from the caudal vein before the first immunization and 7 days after each injection. For the following procedure animals were anesthetized with isofluorane (Cristália, Brazil) in accordance with the recommendations of the *Colégio Brasileiro de Experimentação Animal*. Total serum was harvested 14 days after the last immunization, aliquoted and stored at -80°C. The experimental protocol was approved in institutional revision board (CEUA nº 031/2014)

ELISA assay

The IgG response against Mbp-ORF2p or ORF2p antigens were evaluated by ELISA. ELISA plates were individually coated with either antigen (10 µg/well) diluted in carbonate buffer. Plates were incubated for 2 h at 37ºC followed by an overnight incubation at 4°C. The wells were washed with 0.5% PBS Tween-20 (PBST), and then blocked with 5% (v/v) skim milk (Sigma) diluted in PBST. Rat serum (1:100) was added to the plates and incubated at 37ºC for 60 min followed by peroxidase-conjugated goat anti-rat IgG (Sigma) for 60 min, diluted according to manufacturer's instructions. After washing the plates,

164 0.002% hydrogen peroxide substrate (TMB, Sigma) was added to the wells and
165 after 15 min the reaction was stopped with sulphuric acid 2 M (Sigma). Plates
166 were read on an ELISA reader at 450 nm (Rosys Anthos 2010, Austria). Each
167 serum was run in duplicate and the ODs were analyzed by GraphPad Software.
168 Student's *t*-test was used to compare the OD of the two groups immunized.
169 Significance was set at $p<0.05$.

170 **HEV genotype 3 experimental infection in piglets and alanine**
171 **aminotransferase quantification**

172 HEV-negative 21 days-old piglets (n=5, Large White × Landrace) were
173 experimentally infected (biosafety level II, Ethic Committee n° 031/2014) with
174 20 g of HEV genotype 3-positive swine stool (10^6copies/ml) (LADTV-
175 IOC/Fiocruz) homogenized with 20 ml cool (4°C) RPMI 1640 (Invitrogen) and
176 centrifuged for 1 hour at 13,000 × g. Supernatant was retrieved, carefully
177 filtered (0.22 μm, *TPP*, Switzerland) and cooled at 4°C. Piglets were
178 anesthetized with 25mg/Kg of ketamine and 1 mg/Kg of xylazine and
179 inoculated with 4 ml intravenously (10^6copies/ml). Animals were clinically
180 followed for 21 days post-inoculation. Blood samples and feces were collected
181 daily.

182 Serum levels of alanine aminotransferase (ALT) were evaluated in a semi-
183 automatic analyzer (TP Analyzer Basic, Thermoplate, China) using commercial
184 kits (Labtest Diagnóstica S.A., Brazil). Tests were carried out in duplicate.

185 The experimental protocol was approved in institutional revision board (CEUA
186 nº 031/2014).

187

188

Antigenicity analysis

Recombinant proteins and supernatant from washed feces were analysed by 12% SDS-PAGE gel and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, CA, USA). Membrane was blocked with PBST supplemented with 5% skim milk (Sigma) for 1 hour at room temperature. Serum of rat inoculated with ORF2p (rat anti-ORF2p) or serum from *Cynomolgus* monkey experimentally infected with HEV genotype 3 (LADTV-IOC/Fiocruz) were used as primary antibody, diluted 1:400 and 1:100 in 1% skim milk PBST, respectively. These antisera were incubated at room temperature for 1 hour followed by 3 washes with PBST. The membranes were incubated with secondary antibody conjugated with peroxidase, diluted 1:1,000 (goat anti-rat IgG, Sigma) or 1:5,000 (goat anti-human IgG-HRP, Santa Cruz Biotecnology's, USA), respectively. Proteins were detected adding revealing buffer (HRP Color Development Solution, 4CN), which was prepared following the manufacturer's instruction (Bio-Rad, USA).

Dot blot detection of HEV genotype 3

Stool samples of HEV genotype 3-infected piglets (1 g) were homogenized with 1 ml RPMI 1640 (Invitrogen) and centrifuged for 1 hour at 13,000 × *g*. Supernatant was harvested and concentrate using centrifugal filters (Amicon Ultra 30kDa, Merck Millipore, Germany) according to manufacturer instructions. Two microliters (2µl) of concentrate sample was added to nitrocellulose membrane (Bio-Rad) immobilized in into the Hybri-Slot™ apparatus (Gibco, USA), blocked and washed as described above. Primary antibody (1:400 rat anti-ORF2p) was added and incubated for 1 hour at room temperature. The membrane was washed 3 times; secondary antibody (goat anti

214 rat IgG-HRP, Sigma) diluted 1:1,000 was added and incubated for 1 hour. After
215 being washed 3 times the membrane was revealed as describe for Western blot.
216

Results

Recombinant ORF2 C-terminal nucleotide sequence diversity

The C-terminal portion of the HEV genotype 3 ORF2 was amplified using the primers described in this study (orf-2F and orf-2R) and then aligned with HEV genotype 3 genomes from human and swine isolated in different countries such as Spain, France, Germany, Hungry, Japan, China, Thailand and Brazil. The 801 nucleotides fragment was found highly conserved, with blue squares representing 100% homology against the other 8 sequences analyzed (Fig. 1).

ORF2 cloning and expression

The ORF2p fragment was cloned into a pET20 expression vector to obtain a pET20-His-Mbp-TEV-ORF2p construct. Mbp-ORF2p was expressed in *E. coli* ER2566 bacteria strain and the highest concentration of recombinant protein (10 mg purified protein/L bacteria growth) was achieved with 0.4 mM IPTG stimulation overnight. The purified Mbp-ORF2p and ORF2p recombinant protein (obtained after TEV protease enzymatic digestion of the Mbp-ORF2p fusion protein) was analyzed by SDS-PAGE and the molecular weight was estimated in 74 kDa and 30 kDa respectively (Fig. 2).

Immunogenicity and antigenicity

All rats immunized with the fusion (Mbp-ORF2p) or recombinant (ORF2p) protein seroconverted after the first immunization. To evaluate the homologous antigen immune response, plates were coated with each antigen used in the immunization process. Both antigens induced high antibody titers (Fig. 3A). Rats immunized with Mbp-ORF2p protein had higher antibody titers ($p<0.01$) after the second and third immunization compared to the ORF2p-immunized group (Fig. 3A). Similar tendency was observed when the serum obtained from

242 rats immunized with Mbp-ORF2p antigen were tested in the ELISA plate
243 coated with the ORF2p recombinant protein (Fig. 3B).
244 The antigenicity of the ORF2p recombinant protein was demonstrated by
245 western blot (Fig. 4). Rat anti-ORF2p antibodies (Fig. 4B) as well as monkey
246 anti-HEV antibodies (Fig.4A) were capable to recognize the ORF2p and Mbp-
247 ORF2p recombinant proteins. HEV genotype 3 from swine stool was detected
248 by rat anti-ORF2p (Fig. 4B, lane 7). Mbp-ORF2p (lanes 2 and 4) displayed two
249 bands: the lower band corresponded to the recombinant ORF2p (30 kDa) and
250 the upper band corresponded to the fusion protein (≈74 kDa). Two intermediate
251 bands could be observed close (73 kDa) and about 50 kDa to fusion protein due
252 to proteolysis. The specificity of the rat anti-ORF2p antibodies was
253 demonstrated in lanes 5 and 6.

254 ***In vivo* viral amplification**

255 Five HEV genotype 3-negative piglets were experimentally infected with HEV
256 genotype 3-positive swine feces (confirmed by PCR). Only piglets III and IV
257 presented minor subclinical manifestations 14 days post-inoculation (data not
258 shown). As HEV mainly targets the liver, serologic ALT enzyme was studied
259 to monitor hepatic function (Fig. 5). Piglet II presented a rapid increment of
260 ALT enzyme in the bloodstream (from 40 to 80 Units/L after 7 days after-
261 infection), so that the animal was sacrificed at this time. Liver and feces were
262 harvested to proceed with viral detection. ALT enzyme quantification in piglets
263 III and IV indicated a 1.4 and 1.5 fold increase at day 14 and a 1.4 and 2.5 fold
264 increase at day 21 post-infection (Fig. 5). In piglets I and V the level of ALT
265 enzyme was not altered throughout the experiment. With the exception of piglet
266 II, all animals were euthanized 21 days after the first infection and were

267 submitted to necropsy. No visible signs of hepatic or intestinal lesions were
268 encountered.

269 **HEV genotype 3 detection in feces of inoculated pigs**

270 Molecular detection of the virus was performed by PCR. Piglets' feces were
271 investigated daily for virus shedding. HEV genotype 3 was detected in the feces
272 of piglets III, IV and V starting from the 14th day post-infection, while it
273 remained undetected in the stool of piglets I and II (Fig. 6). Likewise, livers
274 from piglets III, IV and V tested positive for HEV genotype 3 after euthanasia
275 but piglets I and II showed no presence of the virus (Fig. 6).

276 The same samples were used to evaluate the capacity of anti-ORF2p antibodies
277 to detect HEV genotype 3, which was confirmed to be present in 3 out of 4
278 piglets (see above). Dot blot assay revealed that after 21 days post-inoculation,
279 our anti-ORF2p antibody was able to detect the virus in stool of piglets III, IV
280 and V (Fig. 7).

281

282

283 **Discussion**

284 Hepatitis E virus infection has been detected worldwide, increasing its
285 toll on public health (Emerson and Purcell 2007; Meng 2013; Purcell and
286 Emerson 2008). Recent data estimates 20 millions new HEV infection every
287 year, 3 millions of which develop hepatitis E clinical symptoms (WHO 2015).

288 Hepatitis E is mainly associated with consumption of contaminated
289 drinking water (Corwin *et al.* 1999; Daniel *et al.* 2004; Escriba *et al.* 2008;
290 Naik *et al.* 1992). Diagnostic of HEV, based on serologic analysis, is
291 performed by commercial kits that have been developed to recognize protein
292 antigens of HEV genotype 1 and 2 (Drobeniuc *et al.* 2010; Herremans *et al.*
293 2007). Putatively zoonotic HEV genotype 3 and 4 are not included in the
294 available commercial kits for diagnosis (Drobeniuc *et al.* 2010; Herremans *et*
295 *al.* 2007), so that their circulation may result under-detected. These genotypes
296 not only represent a threat for human health (Meng *et al.* 2002) but they are
297 also known to be enzootic in swine herds in China (genotype 4), USA
298 (genotype 3) and Brazil (genotype 3) (Meng *et al.* 1999; Vitral *et al.* 2005;
299 Zheng *et al.* 2006).

300 Amongst structural HEV antigens, surface protein ORF2 has been used
301 to develop diagnostic tests and vaccines for humans and animals (Li *et al.*
302 2005; Zhang *et al.* 2009). The C-terminal of ORF2 contains conserved epitopes
303 in all 4 genotypes (Emerson *et al.* 2006; Meng *et al.* 2001). However, studies
304 demonstrated that human and animal sera immune-reactivity is much stronger
305 when directed against genotype-homologous ORF2 and ORF3 (Herremans *et*
306 *al.* 2007; Ma *et al.* 2011).

307 Recent studies demonstrated that HEV genotype 3 not only circulates in
308 swine farms in Brazil (dos Santos *et al.* 2009), but it is also associated with
309 acute cases of human hepatitis E (Lopes Dos Santos *et al.* 2010). However, no
310 commercial kits are available to detect this genotype. Thus, we expressed a 30
311 kDa fragment from HEV genotype 3 ORF2. Our recombinant protein was
312 aligned with several ORF2 HEV genotype 3 public sequences, scoring a 98%
313 homology (Fig. 1). This highly conserved region resulted to be a valid
314 candidate for diagnostic purposes or vaccine design.
315 The necessity to have reasonable amounts of HEV is limited by the lack
316 of an appropriate cell line to use for its multiplication. Many efforts were made
317 to produce a high quantity of recombinant ORF2 with preserved antigenic and
318 immunogenic characteristics. Different systems were developed to obtain a
319 relative large-scale protein production, such as mammal eukaryote or insect
320 cell lines transfected with recombinant baculovirus or vaccinia virus (Jimenez
321 de Oya *et al.* 2012; Jimenez de Oya *et al.* 2009; Robinson *et al.* 1998; Zhang
322 *et al.* 2001; Zhou *et al.* 2006). ORF2 expression in *E. coli* (Zhang *et al.* 2001)
323 and plastids have also been described (Zhou *et al.* 2006). The most efficient
324 system to produce recombinant ORF2 (≈15mg/L growth) was achieved by
325 transfecting Sf-9 (*Spodoptera frugiperda*) insect cell line with a recombinant
326 baculovirus (Robinson *et al.* 1998). In this study we used *E. coli* ER2566 strain
327 to express our Mbp-TEV-ORF2p recombinant protein yielding 10 mg of
328 soluble protein for each liter of culture media. The high solubility of ORF2p
329 may be related to the Mbp as an associated fusion protein. Indeed, Mbp is
330 capable of increasing the solubility of the target protein if compared to other
331 fusion proteins such as glutathione S-transferase or thioredoxin (Fox *et al.*

2003; Kapust and Waugh 1999). A recent study expressed a HEV genotype 1 ORF2 truncated protein in *E. coli* using pET30a expression vector without Mbp obtaining 1 mg/L of purified protein (Farshadpour *et al.* 2014). The difference of the yield achieved may be at least in part dependent on the choice of using Mbp to increase expression and solubility of ORF2 recombinant protein.

Both Mbp-ORF2p and ORF2p were immunogenic in rats. However, the fusion protein (Mbp-ORF2p) proved to stimulate more IgG synthesis than ORF2p (Fig. 3) perhaps by exposing additional epitopes. Antibodies from swine, human or *Cynomolgus* monkey did not recognize Mbp, making of this fusion protein a candidate in the development of diagnostic tools and vaccine (data not shown). Importantly, rat anti-Mbp antibodies did not recognize any microorganism or molecule in feces, blood and liver from HEV-infected animals nor from HEV-negative bovines and humans (data not shown). An antigenicity property of the ORF2p proteins was confirmed by western blot (Fig. 4), where rat anti-ORF2p antibodies recognized both ORF2p and native HEV ORF2 protein.

In line with previous studies (Halbur *et al.* 2001; Meng *et al.* 1998), our HEV genotype 3-infected pigs maintained showed asymptomatic clinical profile (an overall healthy clinical profile without fever, prostration, vomit or jaundice). Even so, ALT enzyme was altered in some of the animals. Thus, in this study ALT enzyme monitoring did not represent a valid parameter to discriminate an ongoing HEV infection. One could speculate that as natural HEV reservoirs, pigs do not develop major clinical symptoms and hepatic alteration or that the time frame was too short to observe a significant ALT

increase *in vivo*. Absence of this enzyme alteration was previously reported in swine infected with human HEV (Halbur *et al.* 2001) and discrete elevation was reported in *Cynomolgus* monkey infected with swine HEV genotype 3 (de Carvalho *et al.* 2013).

Since the first epidemiologic report of swine HEV infection in Brazil was published (Lyra *et al.* 2005), many studies have focused on hepatitis E both in human and animals (dos Santos *et al.* 2009; Passos-Castilho *et al.* 2015). It has been previously shown that swine or human HEV genotype 3 can cross species barrier infecting *Cynomolgus* monkeys (de Carvalho *et al.* 2013). In our study, piglets were inoculated with swine infected fecal material that naturally represents one source of infection. Stool analysis 14 days after the inoculation indicated that 3 out of 4 piglets tested positive for HEV genotype 3 (PCR confirmation), indicating that the animals were indeed infected and potentially able to spread the virus despite appearing clinically normal. Of note, this result was in line with previous findings where HEV was detected in nasal and rectal swab 2 weeks post-inoculation and animals seroconverted 4 to 8 weeks after the infection (Meng *et al.* 1998). Importantly, in our study, rat anti-ORF2p antibodies recognized both the recombinant ORF2p and the HEV genotype 3 isolated from fecal material. The time frame for this detection was 21 days post-infection, as previously reported (Halbur *et al.* 2001; Meng *et al.* 1998; Meng *et al.* 1998).

In conclusion, we developed a workflow to produce a recombinant HEV genotype 3 ORF2 protein with a high yield and intact antigenic properties. Also, rat antibodies produced against ORF2p specifically recognize the recombinant protein and HEV genotype 3 virus, indicating that ORF2p is a

382 good candidate for developing serologic tests and possibly vaccine for human
383 and animals.

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Conflict of interest

No conflict of interest declared.

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597 **Legends to the figures**

598

599 **Figure 1.** ORF2p multiple alignment against 8 HEV genotype 3 from human
600 (H) or from swine (S). Sequences within blue box represent 100% homology,
601 which cover 98% of the whole analyzed fragment (251 aa). Red or pink blocks
602 represent one or more different residues among the sequences.

603

604 **Figure 2.** SDS-PAGE analysis of HEV ORF2p recombinant protein. Line 1,
605 molecular weight markers; Line 2, purified ORF2p recombinant protein
606 (30kDa); Line 3, Mbp-ORF2p fusion protein (72kDa).

607

608 **Figure 3.** Indirect ELISA quantifying serologic rat IgG. Mbp-ORF2p (circle)
609 and ORF2p (square) immunized rat sera were collected before and 21, 42 or 56
610 days post-infection. Both sera were tested against homologous antigen (A) or
611 ORF2p (B). Student's paired *t* test, * $p<0.05$, ** $p<0.01$.

612

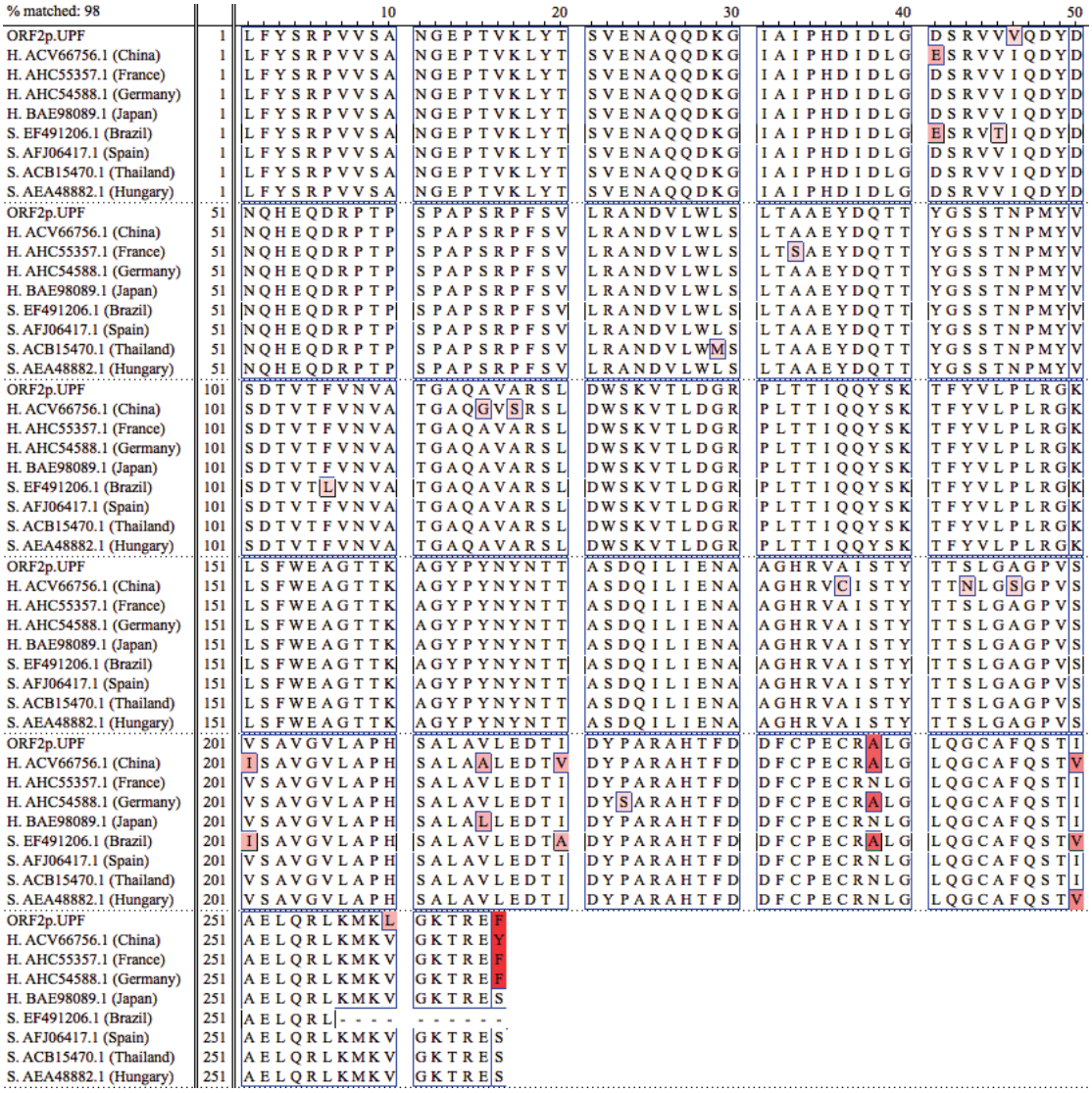
613 **Figure 4.** Protein analysis of ORF2p, Mbp-ORF2p and HEV genotype 3 ORF2.
614 ORF2p and Mbp-ORF2p recombinant protein were detected with monkey anti-
615 HEV genotype3 (panel A, line 1 and 2, respectively). Panel B: ORF2p, Mbp-
616 ORF2p and native HEV were detected with rat anti-ORF2p (lines 3, 4 and 7,
617 respectively). Extract of *E. coli* ER2566 transformed with empty pET20a (panel
618 B, line 5) and feces from piglet I before the infection (panel B, line 6) were used
619 as negative control for rat anti-ORF2p antibody. Stool from piglet IV collected
620 21 days post-infection (panel B, line 7) were detected with rat anti-ORF2p as
621 primary antibody. MW: molecular weight.

Figure 5. Hepatic ALT enzyme survey. Swine hepatic ALT enzyme was quantified during the experiment. Dashed red line represents the mean of ALT values before the infection.

Figure 6. ORF2 PCR to confirm HEV infection. HEV infection was confirmed by PCR detecting ORF2 (801bp). Line 1-5: feces from piglets I-V. Line 6-10: liver from piglets I-V, respectively. Line 11: bovine stool (negative control).

Figure 7. HEV genotype 3 detection by dot blot. Feces from experimentally infected piglets were assessed by dot blot assay. Right panel: stool from animals I, III, IV and V were analyzed at day 0 (above dashed line) or 21 days post-infection (below dashed line). Left panel: ORF2p recombinant protein (C1) and swine HEV genotype 3-positive stool (C2) were used as positive control; swine HEV genotype 3-negative (C3) and bovine (C4) feces were used as negative control. Rat anti-ORF2p polyclonal antibody was used as primary antibody.

Figure 1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S. ACB15470.1 (Thailand) 1 LFYSRPVVSA NGEPTVKLYT SVENAQQDKG IAIPHDIDLG DSRVVIQDYD
S. AEA48882.1 (Hungary) 1 LFYSRPVVSA NGEPTVKLYT SVENAQQDKG IAIPHDIDLG DSRVVIQDYD
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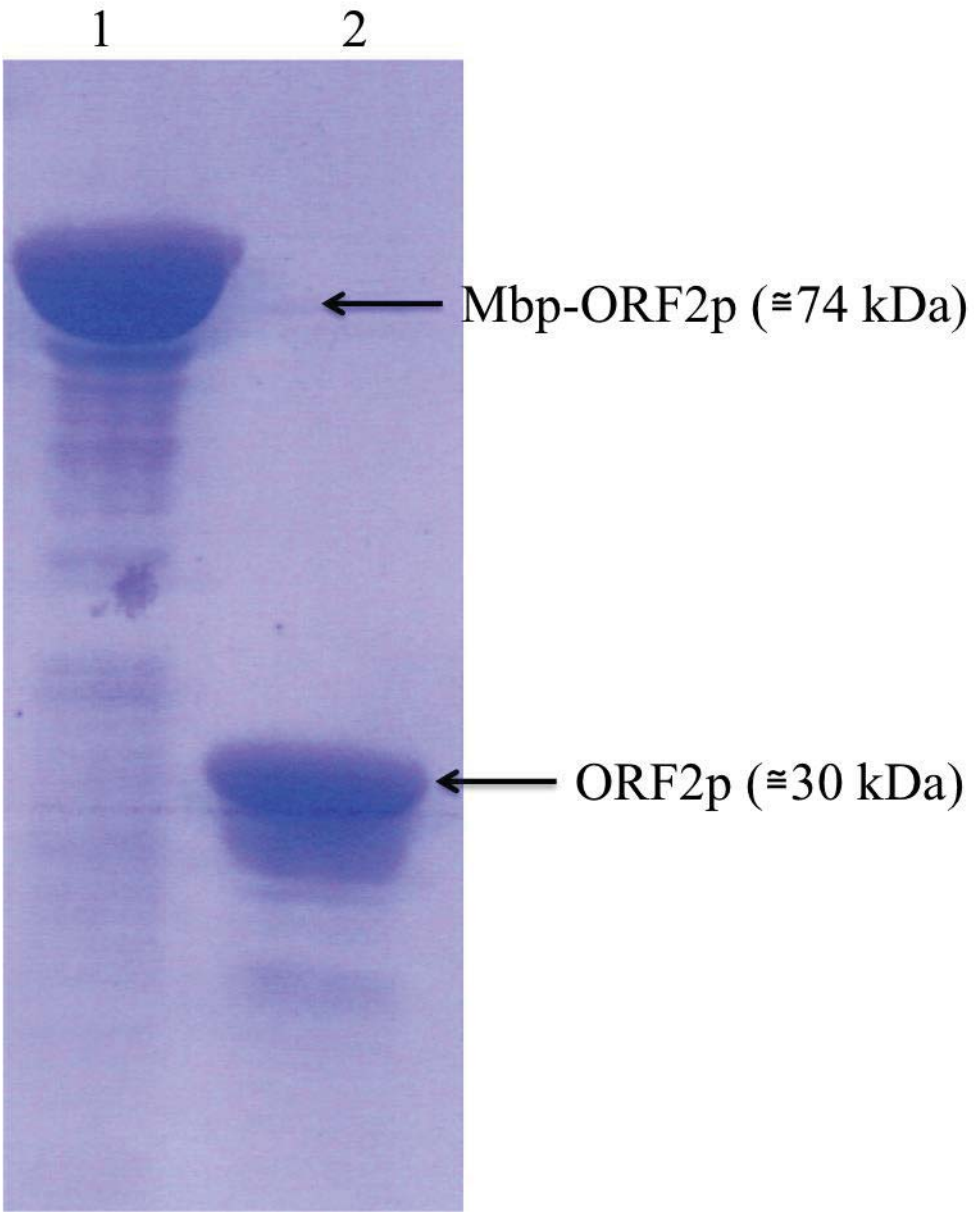
Figure 2



651 **Figure 3**

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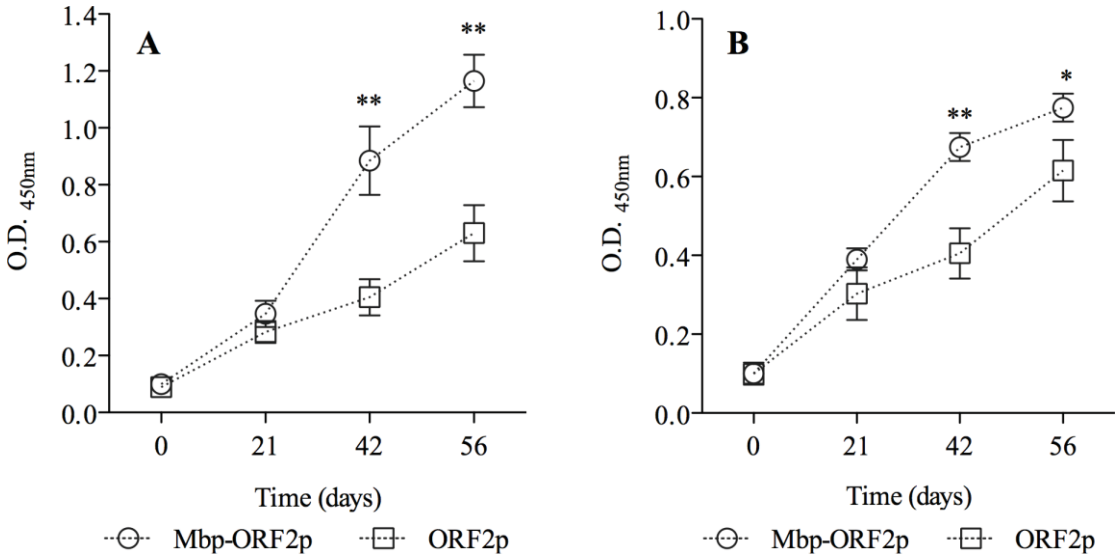

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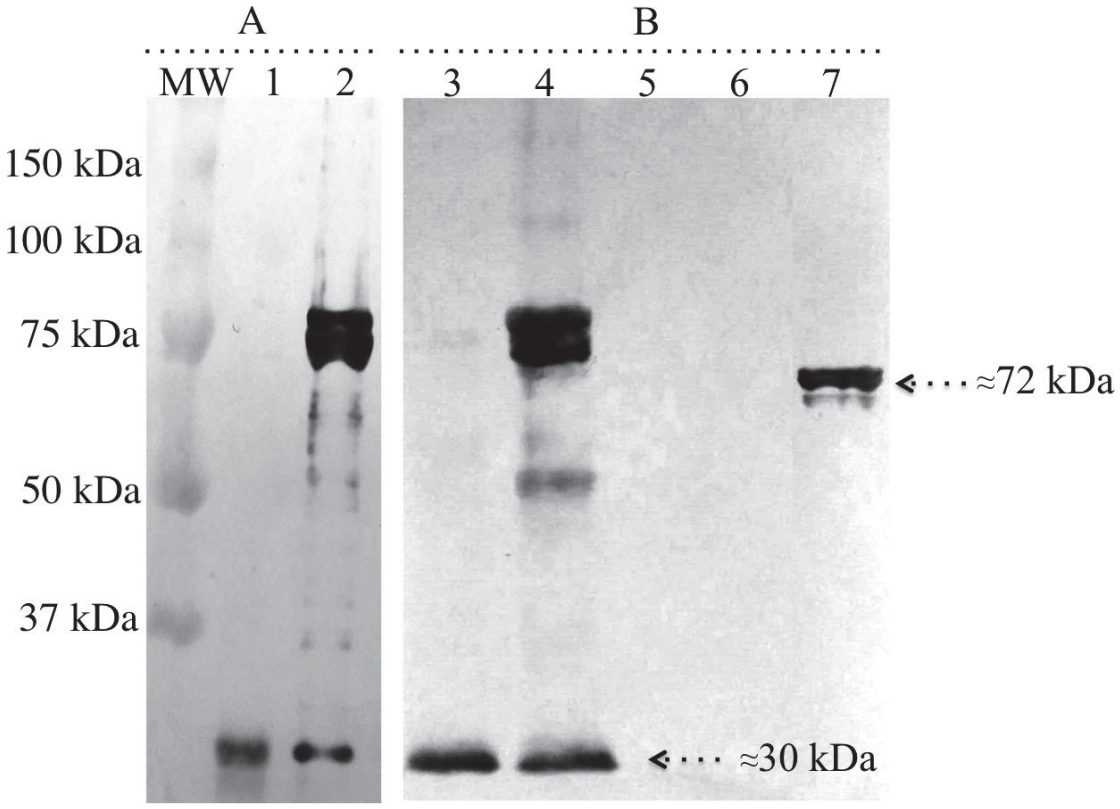

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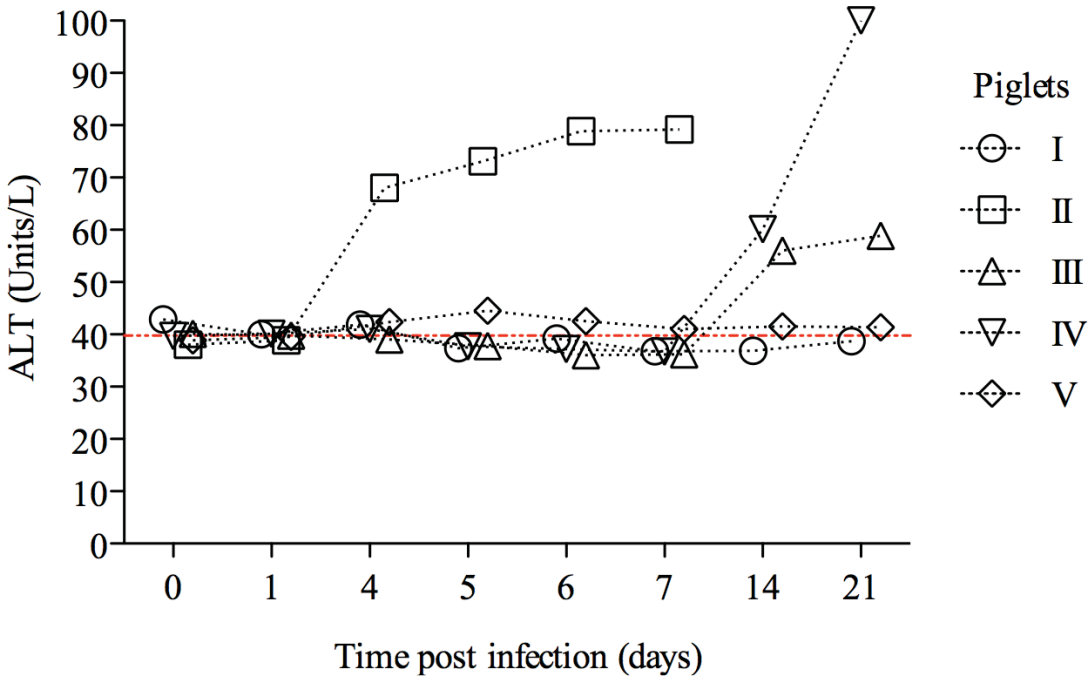

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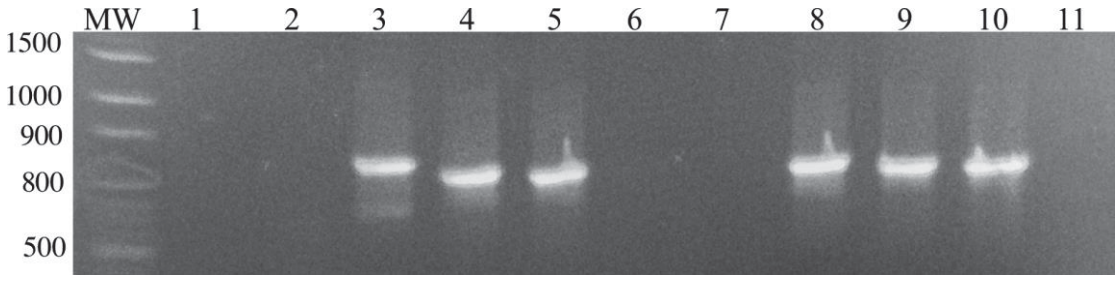
658 **Figure 6**



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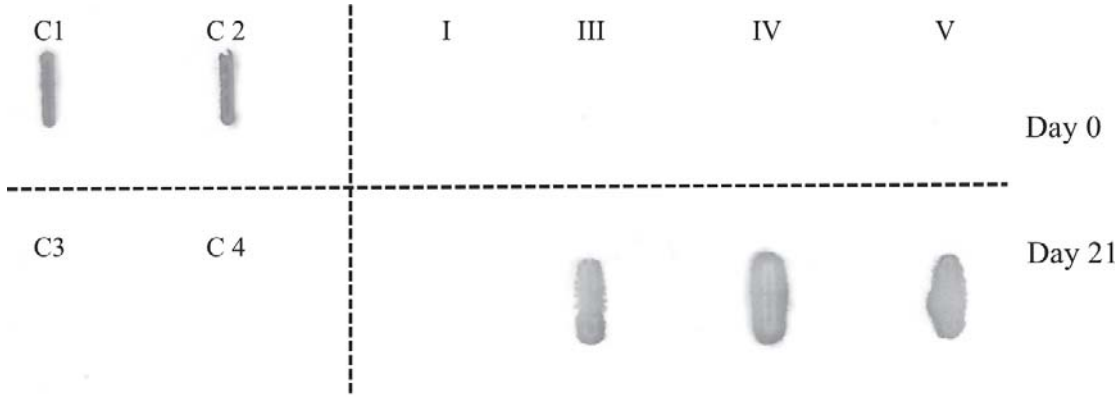
660 **Figure 7**



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4. CONCLUSÕES

A Hepatite Viral E é uma doença infectocontagiosa pouco conhecida no Brasil. Nosso estudo, pioneiro no Brasil, apresenta o desenvolvimento de um antígeno recombinante derivado do Vírus da Hepatite E genótipo 3, o qual poderá ser utilizado tanto no desenvolvimento de testes diagnósticos como também na formulação de vacinas contra este importante patógeno. Assim concluímos que:

Primeira:

A sequência nucleotídica codificante do extremo C-terminal da proteína ORF2 do Vírus da Hepatite E genótipo 3 (isolado brasileiro) é altamente conservada, apresentando homologia de 98% quando comparado com outras sequências do HEV genótipo 3 procedente de humanos e suínos.

Segunda:

O sistema de expressão baseado no vetor pET20a transformado em células de *Escherichia coli* cepa ER2566 demostra-se adequado para produzir a proteína recombinante ORF2p de forma solúvel e em alta concentração.

Terceira:

A proteína recombinante ORF2p além de ser imunogênica em ratas Wistar conserva um repertório de epítopos equivalentes aos encontrados na proteína ORF2 nativa, evidenciando sua qualidade antigênica.

5. CONSIDERAÇÕES FINAIS

Hepatite E é uma doença viral emergente de grande importância em saúde pública, diferente das demais hepatites, a hepatite E é zoonótica, podendo ser transmitida por várias espécies animais.

Países com alta produção de suínos, como China, Estados Unidos, Espanha, França e Alemanha, chamam a atenção para a presença e circulação dos genótipos zoonóticos do vírus da hepatite E, e ao mesmo tempo, notificam inúmeros casos de hepatite em humanos produzidas por estes genótipos.

Atualmente, o Brasil detém o quarto principal rebanho de suínos do mundo, ocupando lugar de destaque entre os principais exportadores desta proteína animal. Contudo, estudos soroepidemiológicos relacionados com o HEV são escassos e, consequentemente, desconhecemos a real importância desta doença em nosso país.

Neste trabalho, descrevemos o desenvolvimento de um antígeno recombinante, derivado da proteína ORF2 do HEV gt3 que permitirá, por um lado, realizar futuros estudos sorológicos em humanos e em suínos através do desenvolvimento de um teste de diagnóstico customizado com o antígeno descrito e, por outro, desenvolver de uma vacina de subunidade para suínos, com potencial de eliminar a infecção da principal espécie animal considerada reservatório do principal genótipo do vírus da hepatite E circulante no Brasil.

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