BMC Microbiology

RESEARCH ARTICLE Open Access

Hepadnavirus detected in bile and liver samples from domestic pigs of commercial abattoirs

Yasmine Rangel Vieira[1*], Debora Regina Lopes dos Santos[2], Moyra Machado Portilho[3], Carlos Eduardo Pereira Velloso[1], Marcia Arissawa[4], Livia Melo Villar[3], Marcelo Alves Pinto[1] and Vanessa Salete de Paula[1]

Abstract

Background: Preliminary studies showed the prevalence of a virus similar to human hepatitis B virus (HBV-like) in swine from farms in China and the molecular evidence of Hepadnavirus infection in domestic pigs herds in Brazil. In this study, we genetically characterize the swine Hepadnavirus strains in swine from slaughterhouses located in certified abattoirs from Rio de Janeiro State, Brazil and evaluate its hepatotropic potential.

Results: Bile and liver samples from swine were positive for partial genome amplification (ORF S and ORF C), direct sequencing and viral load quantification. Sequencing of the gene encoding the surface antigen allowed classification of Hepadnavirus into genotypes, similar to HBV genotype classification. Indirect immunofluorescence confirmed the presence of HBsAg antigen in liver tissue sections.

Conclusions: So far our data suggest that commercial swine house an HBV-like virus and this relevant finding should be considered in studies on the origin and viral evolution.

Keywords: Hepadnavirus, Brazil, Commercial swine, Bile, Liver, Genotyping, Immunofluorescence

Background

Hepatitis B virus (HBV) is a dual polarity and partially double-stranded enveloped DNA virus of *Hepadnaviridae* family [1]. The agent can be transmitted by sexual, perinatal and percutaneous means [2], and is considered a major cause of acute and chronic liver disease, that may progress to cirrhosis and hepatocellular carcinoma [3]. Beyond the prototype member that infects humans and non-human primates (chimpanzees, gibbons, gorillas, orangutans and woolly monkeys), *Hepadnaviridae* family also houses HBV-related viruses circulating in mammalian hosts, like woodchuck (WHV) and squirrels (GSHV/ASHV), and avian hosts, like ducks (DHBV), geese (GHBV), herons (HHBV), and storks (STHBV) [4].

A virus similar to HBV has been diagnosed by serology (HBsAg, anti-HBs, anti-HBc) in swine herds [5] and chickens flocks [6] from China, and in domestic pig herds in Brazil [7]. Moreover, positive molecular diagnosis was demonstrated for the first time in swine from Brazil [7] and in chickens flocks from China [6]. Similarity with human HBV (90.8-96.3%) was confirmed for swine strains by phylogenetic analysis and by cross reactivity in non-host specific commercial serological assays. However, the partial nucleotide sequencing (360 bp) was equivalent to about 11.2% of full-length HBV genome. And even for chickens, despite the high percentage of similarity with HBV (92.2-97.9%), the short length of amplified product in both cases limits a conclusion.

Therefore, we performed the current study to improve the molecular characterization of Hepadnavirus circulating in swine from abattoirs in Brazil, revealing if there is evidence that pigs destined for human consumption might act as a potential new reservoir or host for a virus HBV-similar.

Methods

This study was approved by the Institutional Committee for Ethics in the Use of Research Animals (CEUA-Fiocruz: PO 0132/01). On December 2008, a total of 36 bile and liver samples were collected from domestic pigs *Sus scrofa* (aged > 5 months), breed Large white, from three slaughterhouses located in Petrópolis (SPET), Itaocara (SITC) and Itaperuna (SITP) (North and Hill region of Rio de

* Correspondence: yasminerangel@ig.com.br
[1]Laboratório de Desenvolvimento Tecnológico em Virologia, Pavilhão Hélio e Peggy Pereira – 2° andar - sala B220, Instituto Oswaldo Cruz, FIOCRUZ, Av. Brasil, n° 4365, Manguinhos - RJ, Cep: 21045-900 Rio de Janeiro, RJ, Brasil
Full list of author information is available at the end of the article

BioMed Central

© 2014 Vieira et al.; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

Janeiro State, Brazil). Commercial establishments are submitted to controlled inspection by an official agency of Rio de Janeiro State. All animals from the three abattoirs were classified healthy and approved for slaughter and further commercialization according to inspect evaluation criteria. Swine from each abattoir were acquired from distinct pig farms suppliers. The samples were collected during the evisceration process under sanitary requisites determined by regulations of Animal Sanitary Protection Agency in Rio de Janeiro State (ASPA).

Five milliliters of bile were collected by vaccum-punction through the gallbladder wall with sterile syringe; and 500 mg of liver samples were collected with Medblade® bisturi blades. All samples were stored in Nalgene® cryogenic vials and immediately frozen in dry ice. At the laboratory, bile samples were stored at -80°C and liver samples in liquid nitrogen until analysis. Viral DNA was extracted from bile and liver samples using DNA Purification Kit (QIAamp DNA Mini Kit, Qiagen®) according to Moricz et al, 2010 [8] and was concentrated to a final volume of 25 uL. Extracted DNA was analyzed by semi-nested PCR (PS1-S2 and PS1-SR) specific for open reading frame (ORF S) of HBV - as previously established for the first round of amplification [9], and for the second round [10] - and by PCR specific for core gene of HBV – as previously established [11]. Direct sequencing of amplicons was performed to identify the sequence amplified.

After molecular diagnosis, in order to quantify the viral load DNA samples were evaluated in duplicate by Real Time PCR using TaqMan® method. The Real Time PCR assay was performed for pre-S2/S region as previously established [12], using the following primers pair and fluorescent probe: forward primer (5'-GAATCCTCACA ATACCGCAGAGT-3'), reverse primer (5'-GCCAAGA CACACGGGTGAT-3'), and probe (5'-FAM-AAGTCC ACCACGAGTCTAG-NFQ/MGB-3') (Life Technologies®, Applied Biosystems). HBV plasmid serially diluted from 5×10^7 to 5×10^1 copies/ µL was used as HBV standard PCR template. The amplification was analyzed using the software Applied Biosystems 7500.

Indirect immunofluorescence assay was also performed in samples of liver tissue cryosectioned at 4 µm. Liver biopsies were stained with anti-HBs mouse monoclonal primary antibody specific for human HBsAg (19-CC6/ CG2 Lot 100830S019, Biomanguinhos, Fiocruz, Rio de Janeiro, Brazil) at the dilution 1:50 overnight at 4°C, followed by Alexa Fluor 488 conjugated goat anti-mouse secondary antibodies (Molecular Probes®) at the dilution 1:400 for 1 h at 37°C. Liver sections were counterstained with Evans Blue (1: 20,000) and DAPI (Molecular Probes®). Images of

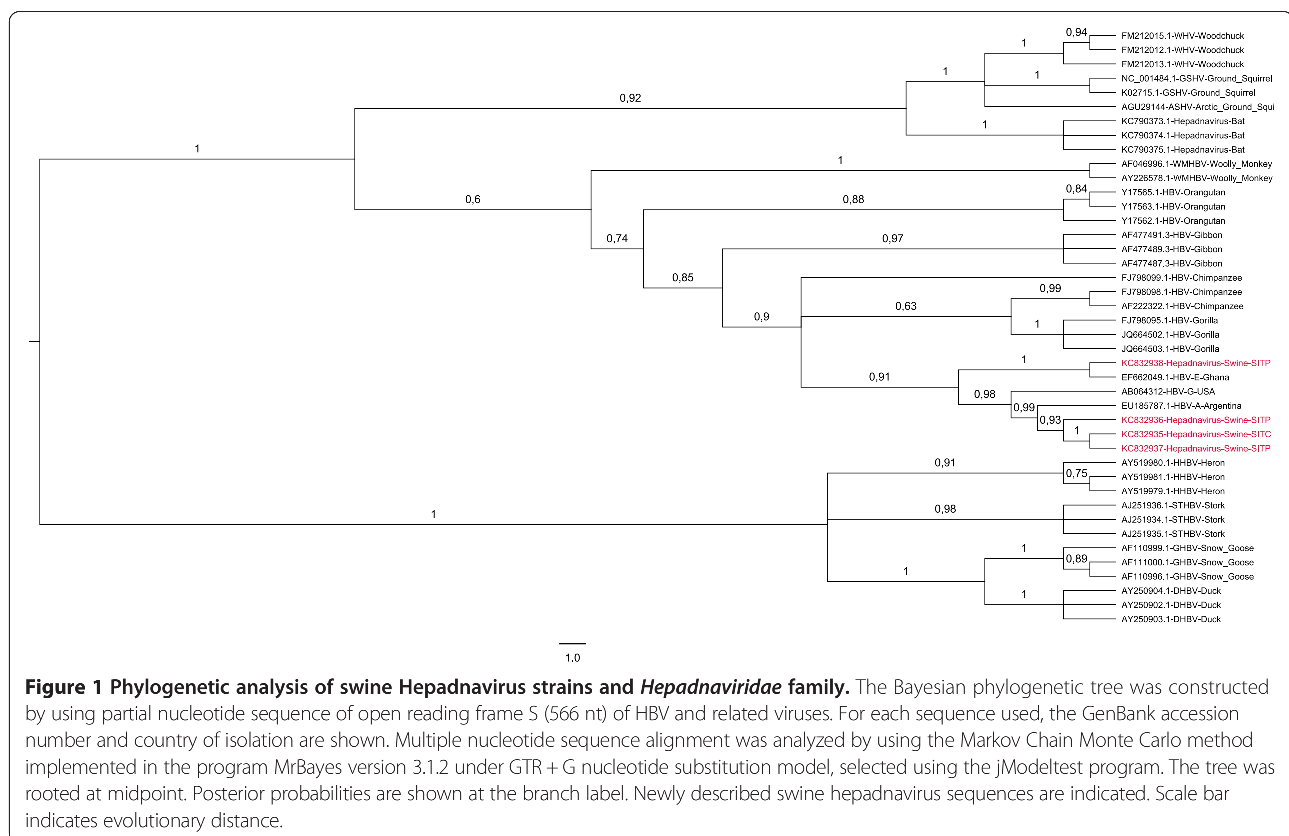

Figure 1 Phylogenetic analysis of swine Hepadnavirus strains and *Hepadnaviridae* family. The Bayesian phylogenetic tree was constructed by using partial nucleotide sequence of open reading frame S (566 nt) of HBV and related viruses. For each sequence used, the GenBank accession number and country of isolation are shown. Multiple nucleotide sequence alignment was analyzed by using the Markov Chain Monte Carlo method implemented in the program MrBayes version 3.1.2 under GTR + G nucleotide substitution model, selected using the jModeltest program. The tree was rooted at midpoint. Posterior probabilities are shown at the branch label. Newly described swine hepadnavirus sequences are indicated. Scale bar indicates evolutionary distance.

the green fluorescent HBsAg-positive liver cells were observed and photographed using a Confocal Scanning Laser Microscope equipped with camera (Nikon® Instruments, Model C2, Inc., New York, USA).

Results

Hepadnavirus-DNA was detected in bile and liver samples from about 11.11% pigs (4/36). All samples from Petrópolis (0/9) were tested negative. Hepadnavirus-DNA was detected in both samples types from 1 animal in Itaocara (1/10) and from 3 animals in Itaperuna (3/17). These samples showed a positive result for the semi-nested PCR specific for HBV ORF S (1,100 bp) [GenBank: KC832935, GenBank:KC832936, GenBank:KC832937, GenBank:KC832938], and 3 of them also for the PCR specific for core gene (431 bp) [GenBank:KF859967, GenBank:KF859968, GenBank:KF859969]. The sequences found in bile and liver of the same animal matched. Phylogenetic reconstruction using partial nucleotide sequences of ORF S (566 nt) showed a close relationship of Hepadnavirus strains from pigs and Hepadnavirus nucleotide sequences from non-human primates (from 84.8 to 96.1%), bats (from 68.9 to 75.6%), rodents (from 70.5 to 71.8%) and birds (from 45.8 to 48.6%) (Figure 1, Table 1).

Genetic distances calculated with nucleotide sequences of ORF S (972 nt) showed filogenetic distance between the virus detected in Brazilian domestic pigs and human samples from significant alignment listed by NCBI Blast [GenBank:JF784228, GenBank:JF784230, GenBank: JF784231, GenBank:JF784234, GenBank:EF662027-49, GenBank:DQ060826, GenBank:DQ060829] ranging from 98.9-99.7%. These data also revealed that distinct strains might be responsible for the infection in domestic pigs (Figure 2). Comparing to human HBV strains previously genotyped, swine samples were assigned to two different genotypes groups. Three of them were close to samples from genotype A, the most prevalent genotype in Brazil [13]. And, another was similar to samples that belong

Table 1 Nucleotide identity between Hepadnavirus strains from pigs and other species

Species	Accession Number GenBank	Swine Hepadnavirus			
		KC832935	KC832936	KC832937	KC832938
Chimpanzee (HBV)	FJ798099.1	0.950	0.950	0.951	0.961
	AF222322.1	0.937	0.937	0.939	0.948
Gibbon (HBV)	AF477494.2	0.939	0.939	0.940	0.953
	AF274499.2	0.918	0.918	0.920	0.933
Orangutan (HBV)	Y17562.1	0.916	0.918	0.918	0.929
	Y17559.1	0.911	0.913	0.913	0.926
Gorilla (HBV)	AJ131567.1	0.946	0.946	0.948	0.951
	JQ664502.1	0.942	0.942	0.944	0.948
Woolly Monkey (WMHBV)	AF046996.1	0.850	0.848	0.851	0.848
	AY226578.1	0.850	0.848	0.851	0.848
Bat (Hepadnavirus)	KC790378.1	0.689	0.689	0.691	0.695
	KC790376.1	0.755	0.753	0.756	0.755
Woodchuck (WHV)	FM212013.1	0.707	0.705	0.709	0.707
	FM212009.1	0.716	0.714	0.718	0.714
Squirrel (ASHV/GSHV)	AGU29144	0.709	0.709	0.710	0.712
	K02715.1	0.709	0.709	0.710	0.716
Heron (HHBV)	AY552597.1	0.474	0.477	0.476	0.481
	AY552595.1	0.483	0.484	0.484	0.486
Stork (STHBV)	AJ251937.1	0.464	0.466	0.466	0.471
	AJ251935.1	0.464	0.466	0.466	0.468
Snow Goose (GHBV)	AF110999.1	0.458	0.461	0.460	0.461
	AF110997.1	0.461	0.465	0.463	0.465
Duck (DHBV)	AY494851.1	0.467	0.470	0.468	0.461
	M21953.1	0.473	0.476	0.475	0.476

Legend: The nucleotide identity matrix was constructed by using partial nucleotide sequence of open reading frame S (566 nt) of HBV and related viruses. For each sequence used, the GenBank accession number and specie infected are shown. Newly described swine hepadnaviruses sequences are indicated.

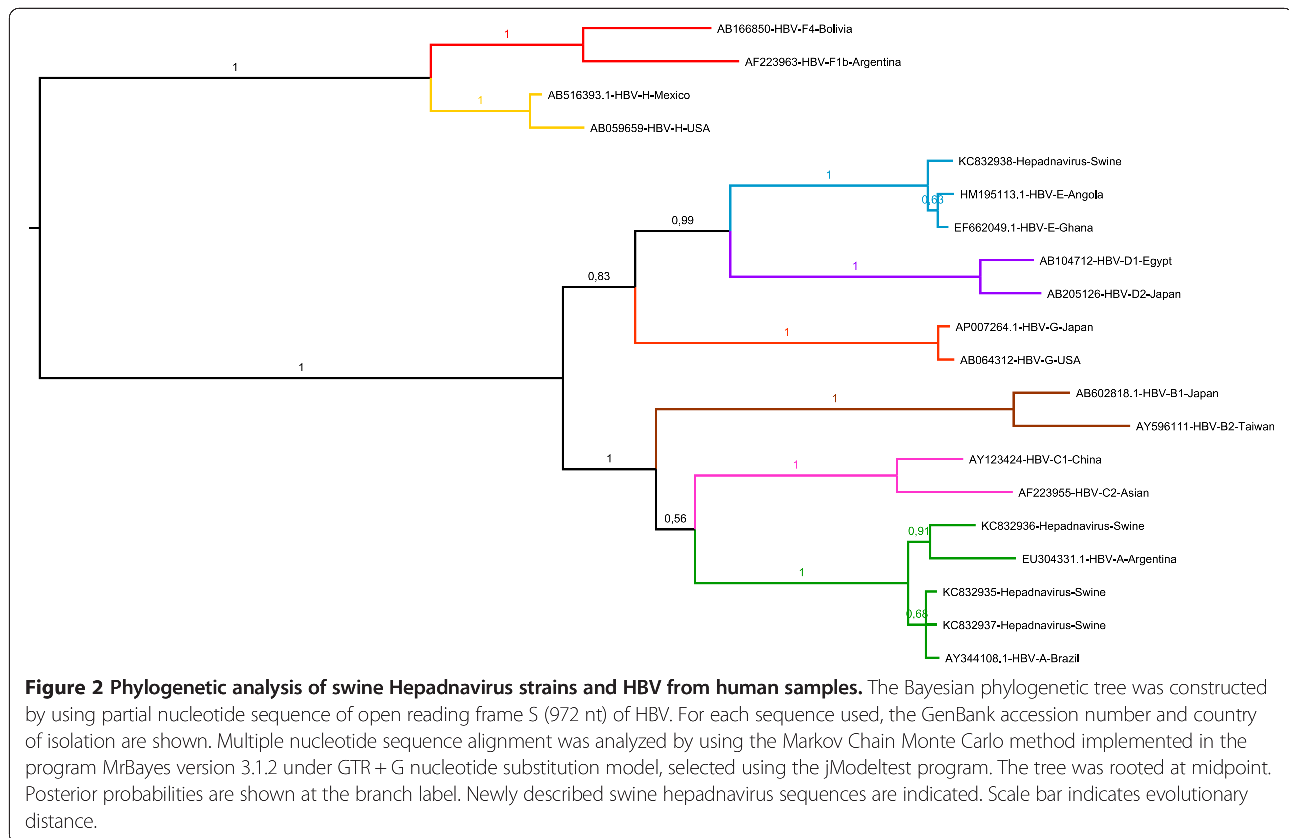

Figure 2 Phylogenetic analysis of swine Hepadnavirus strains and HBV from human samples. The Bayesian phylogenetic tree was constructed by using partial nucleotide sequence of open reading frame S (972 nt) of HBV. For each sequence used, the GenBank accession number and country of isolation are shown. Multiple nucleotide sequence alignment was analyzed by using the Markov Chain Monte Carlo method implemented in the program MrBayes version 3.1.2 under GTR + G nucleotide substitution model, selected using the jModeltest program. The tree was rooted at midpoint. Posterior probabilities are shown at the branch label. Newly described swine hepadnavirus sequences are indicated. Scale bar indicates evolutionary distance.

to genotype E, which is common in western Africa [14]. Genotype A was found circulating in Itaocara and Itaperuna, while genotype E was found exclusively in Itaperuna.

Concerning viral load quantification, a linear relationship was obtained between the cycle threshold (Ct) values and the $\log_{10}$ concentration of the HBV DNA. The regression analysis yielded a correlation coefficient of 0.99. All four bile samples could be quantified. The viral loads ranged from 0.8×10^3 to 1×10^5 copies/mL.

Further, we investigated viral hepatotropism by indirect immunofluorescence analysis using monoclonal anti-HBs antibody in liver biopsies from *Hepdnavirus*-DNA positive animals, and compared the results to the control animals. HBsAg was observed in cell membrane and cytoplasm of sinusoidal lining cells in liver parenchyma and in hepatocytes (Figure 3). No staining was observed for controls. Molecular data and antigenic detection in situ confirm that swine were infected by a Hepadnavirus with hepatotropic behavior, similar to human HBV.

Discussion

Our challenge in this study was to refine the molecular characterization of Hepadnavirus circulating in swine from Brazil, disclosing how close porcine strains are to HBV and to HBV-like viruses sequences described for different hosts, including human. Data presented here showed different strategies for viral detection, including the molecular characterization of complete ORF C and ORF S in bile and liver samples, the genotype classification based on the genotypic classification of HBV, the viral load quantification and antigenic detection in hepatic parenchyma.

This is the first record of molecular characterization of swine Hepadnavirus from bile and liver samples. There are no previous data available in the literature concerning genotypic classification based on complete sequencing of pre-S/S region and viral load evaluation in swine from slaughterhouses.

According to results described previously for Brazilian domestic swine [7], the similarity between pigs and human Hepadnavirus strains was supposed through superficial and preliminary estimates, and cross reactivity in non-host specific commercial serological assays. In this study, molecular results from a larger and more representative fragment of the genome allowed us to calculate an approximate identity not only with HBV from human host, as well as other viruses that comprise the *Hepdnaviridae* family: 98.9-99.7% to human HBV, 93.7-96.1% to Chimpanzee HBV, 91.8-95.3% to Gibbon HBV, 94.2-95.1% to Gorilla HBV, 91.1-92.9% to Orangutan HBV, 84.8-85.1% to Woolly Monkey WMHBV, 68.9%-75.6% to Bat Hepadnavirus, 70.5-71.8% to Woodchuck WHV, 70.9-71.6% to Arctic/ Ground Squirrels ASHV/ GSHV, 47.4-48.6% to Heron

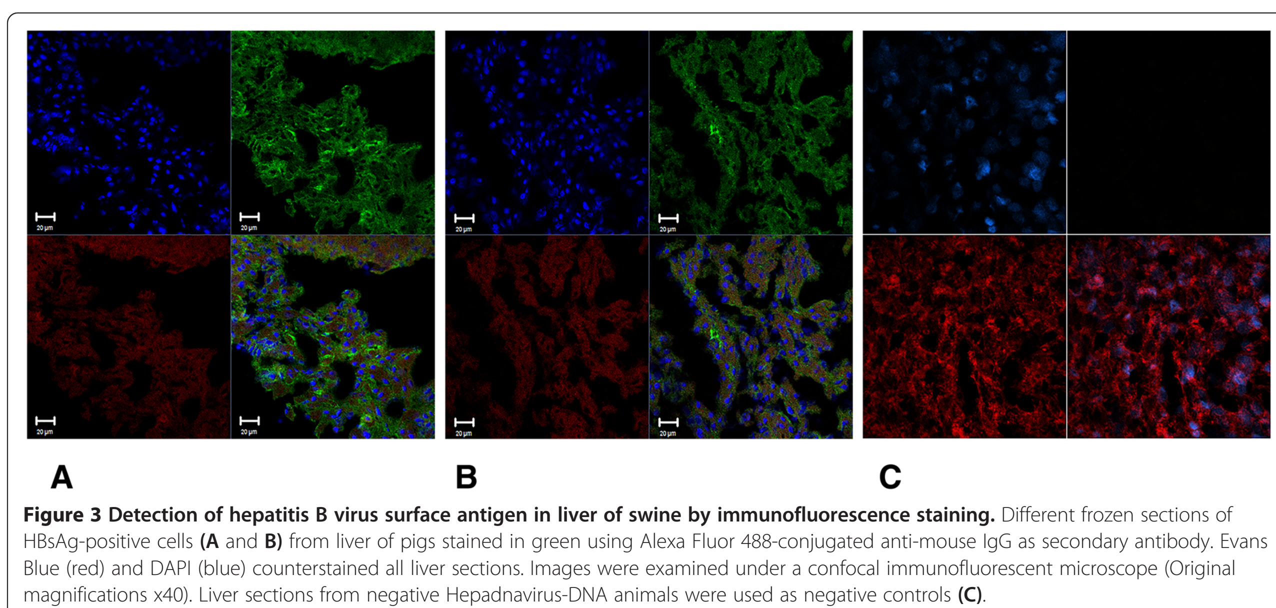

Figure 3 Detection of hepatitis B virus surface antigen in liver of swine by immunofluorescence staining. Different frozen sections of HBsAg-positive cells **(A** and **B)** from liver of pigs stained in green using Alexa Fluor 488-conjugated anti-mouse IgG as secondary antibody. Evans Blue (red) and DAPI (blue) counterstained all liver sections. Images were examined under a confocal immunofluorescent microscope (Original magnifications x40). Liver sections from negative Hepadnavirus-DNA animals were used as negative controls **(C)**.

HHBV, 46.4-47.1% to Stork STHBV, 45.8-46.5% to Snow Goose GHBV, 46.1-47.6% to Duck DHBV.

Genetic distances data are relevant and need to be better evaluated once despite the diversity of hosts, no reservoirs are described for HBV so far [15]. At the same time, as commercial swine are slaughtered at a young age (up to 22 weeks old), the identification of any clinical signs of disease may be hindered notably considering that health inspections are based only in visible lesions present and post-mortem gross macroscopic examinations in animal from abattoirs. No molecular tests are executed. Due to these factors, a follow-up study for longer periods should be considered.

It is noteworthy that investigation of the population of animals from three different commercial herds was performed after a single collection, and 11.11% of swine showed positive molecular diagnosis, in which hepatotropism was also confirmed. These positive animals were distributed in two slaughterhouses which do not share the same pig farm supplier.

The possibility of an eventual vertical transmission can not be ruled out for positive animals from the same slaughterhouse since they came from the same herd. However, as there are distinct breeding sows in a single pig farm, animals destined to the same abattoir may be born from different sows. To consider the risk of vertical transmission, pigs should also share the same genotype in addition to sharing the same geographical origin.

In this study, three positive animals came from Itaperuna. One animal was assigned to genotype E, and two animals shared genotype A. Due to the circulation of both genotypes in swine from the same abattoir, at least two different breeding sows were involved in its respective pig farm supplier. Even though, the risk of vertical transmission for two animals of genotype A cannot be confirmed.

Since the role of bats as reservoirs of zoonotic viruses is now being investigated, including for bat hepadnaviruses [15], further studies are also necessary to extrapolate this reasoning to the pigs analogously. Whether there is a zoonotic ability of swine Hepadnavirus strains and if this virus is able to induce cross-infection in other species are remaining issues. Considering the main routes of HBV transmission, and since personal protective equipment is not often used in slaughterhouses, novel studies are necessary to assess a potential occupational risk of infection by Hepadnaviruses during the management of pigs and their derivatives.

Conclusions

The present study demonstrated the circulation of an emerging virus in swine, similar to Hepadnavirus that causes human infection. Further studies are necessary to disclose the actual role of swine in the viral cycle.

Abbreviations

HBV: Human hepatitis B virus; ORF S: Open reading frame S; ORF C: Open reading frame C; WHV: HBV-related virus of woodchuck; GSHV: HBV-related virus of ground squirrels; ASHV: HBV-related virus of arctic squirrels; DHBV: HBV-related virus of ducks; GHBV: HBV-related virus of geese; HHBV: HBV-related virus of herons; STHBV: HBV-related virus of storks; HBsAg: Surface antigen of HBV; anti-HBs: HBV surface antibody; anti-HBc: Antibody to HBV core antigen; bp: Base pair; CEUA: Committee for Ethics in the Use of Research Animals; ASPA: Animal Sanitary Protection Agency in Rio de Janeiro State; nt: Nucleotide.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

This study was designed by YRV, DRLS, MAP and VSP. YRV performed qualitative molecular assays, sequence analysis, interpreted the data, collaborated to quantitative molecular assays, to immunofluorescence assay and wrote the manuscript. DRLS contributed to the sample collection and helped to write the manuscript. MMP performed quantitative molecular assays. CEPV contributed to the immunofluorescence assay. MA contributed with immunological reagents. LMV contributed with molecular reagents, materials and analysis tools. MAP contributed to the sample collection, coordinated the study and revised the manuscript. VSP coordinated the study and revised the manuscript. All authors read, corrected and approved the final manuscript.

Acknowledgments

This study was supported by Oswaldo Cruz Institute (FIOCRUZ), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ/ E-26/110.607/2014). The authors appreciated the help from Gilberto Vaughan (Centers for Disease Control and Prevention/ CDC) and Leandro Layter Xavier (Universidade do Estado do Rio de Janeiro/ UERJ).

Author details

[1]Laboratório de Desenvolvimento Tecnológico em Virologia, Pavilhão Hélio e Peggy Pereira – 2° andar - sala B220, Instituto Oswaldo Cruz, FIOCRUZ, Av. Brasil, n° 4365, Manguinhos - RJ, Cep: 21045-900 Rio de Janeiro, RJ, Brasil. [2]Departamento de Mircobiologia Veterinária e Imunologia, Universidade Federal Rural do Rio de Janeiro, UFRRJ, Seropédica, RJ, Brasil. [3]Laboratório de Hepatites Virais, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, RJ, Brasil. [4]Laboratório de Tecnologia de Anticorpos Monoclonais, Bio-Manguinhos, FIOCRUZ, Rio de Janeiro, RJ, Brasil.

Received: 21 June 2014 Accepted: 26 November 2014
Published online: 11 December 2014

References

1. Wei Y, Neuveut C, Tiollais P, Buendia MA: **Molecular biology of the hepatitis B virus and role of the X gene.** *Pathol Biol (Paris)* 2010, **58:**267–272.
2. Dienstag JL: **Hepatitis B virus infection.** *N Engl J Med* 2008, **359:**1486–1500.
3. Prange R: **Host factors involved in hepatitis B virus maturation, assembly, and egress.** *Med Microbiol Immunol* 2012, **201:**449–461.
4. Yang J, Xi Q, Deng R, Wang J, Hou J, Wang X: **Identification of interspecies recombination among hepadnaviruses infecting cross-species hosts.** *J Med Virol* 2007, **79:**1741–1750.
5. Li W, She R, Liu L, You H, Yin J: **Prevalence of a virus similar to human hepatitis B virus in swine.** *Virol J* 2010, **7:**60.
6. Tian J, Xia K, She R, Li W, Ding Y, Wang J, Chen M, Yin J: **Detection of hepatitis B virus in serum and liver of chickens.** *Virol J* 2012, **9:**2.
7. Vieira YR, Vieira AA, Ciacci-Zanella JR, Barquero G, do Lago BV, Gomes SA, Silva MFM, Santos DRL, Pinto MA, de Paula VS: **Serological and molecular evidence of Hepadnavirus infection in swine.** *Ann Agric Environ Med*, in press.
8. Moricz A, Melo M, Castro AM, Campos T, Silva RA, Pacheco AM Jr: **Prevalence of Helicobacter spp in chronic cholecystitis and correlation with changes on the histological pattern of the gallbladder.** *Acta Cir Bras* 2010, **25:**218–224.
9. Niel C, Moraes MT, Gaspar AM, Yoshida CF, Gomes SA: **Genetic diversity of hepatitis B virus strains isolated in Rio de Janeiro, Brazil.** *J Med Virol* 1994, **44:**180–186.
10. Motta-Castro AR, Martins RM, Yoshida CF, Teles SA, Paniago AM, Lima KM, Gomes SA: **Hepatitis B virus infection in isolated Afro-Brazilian communities.** *J Med Virol* 2005, **77:**188–193.
11. Lanford RE, Chavez D, Brasky KM, Burns RB 3rd, Rico-Hesse R: **Isolation of a hepadnavirus from the woolly monkey, a New World primate.** *Proc Natl Acad Sci U S A* 1998, **95:**5757–5761.
12. Portilho MM: **Desenvolvimento de testes de detecção e quantificação do vírus da hepatite B em amostras de soro e fluido oral.** *MSc thesis.* Instituto Oswaldo Cruz, Departamento de Virologia. Rio de Janeiro, Brazil; 2013.
13. Becker CE, Mattos AA, Bogo MR, Branco F, Sitnik R, Kretzmann NA: **Genotyping of hepatitis B virus in a cohort of patients evaluated in a hospital of Porto Alegre, South of Brazil.** *Arq Gastroenterol* 2010, **47:**13–17.
14. Kramvis A, Kew MC: **Epidemiology of hepatitis B virus in Africa, its genotypes and clinical associations of genotypes.** *Hepatol Res* 2007, **37:**S9–S19.
15. Drexler JF, Geipel A, Konig A, Corman VM, Van Riel D, Leijten LM, Bremer CM, Rasche A, Cottontail VM, Maganga GD, Schlegel M, Müller MA, Adam A, Klose SM, Carneiro AJ, Stöcker A, Franke CR, Gloza-Rausch F, Geyer J, Annan A, Adu-Sarkodie Y, Oppong S, Binger T, Vallo P, Tschapka M, Ulrich RG, Gerlich WH, Leroy E, Kuiken T, Glebe D, Drosten C: **Bats carry pathogenic hepadnaviruses antigenically related to hepatitis B virus and capable of infecting human hepatocytes.** *Proc Natl Acad Sci U S A* 2013, **110:**16151–16156.

doi:10.1186/s12866-014-0315-2
Cite this article as: Vieira *et al.*: **Hepadnavirus detected in bile and liver samples from domestic pigs of commercial abattoirs.** *BMC Microbiology* 2014 **14**:315.

Submit your next manuscript to BioMed Central and take full advantage of:

- **Convenient online submission**
- **Thorough peer review**
- **No space constraints or color figure charges**
- **Immediate publication on acceptance**
- **Inclusion in PubMed, CAS, Scopus and Google Scholar**
- **Research which is freely available for redistribution**

Submit your manuscript at
www.biomedcentral.com/submit