Daniel da Silva Gonçalves

Prevalence of Hepatitis E virus in Portugal – a study in wild animals and wastewaters

Tese de Mestrado em Análises Clínicas, orientada pela Drª Ana Miguel Duarte Matos Silva
e apresentado à Faculdade de Farmácia da Universidade de Coimbra

Julho 2014

UNIVERSIDADE DE COIMBRA

Prevalence of Hepatitis E virus in Portugal - a study in wild animals and wastewaters

Study made with collaboration of:

Gonçalves D. [1], Vaz J. [2], Donato A. [3], Luxo C. [1], Matos A. [1]

1 – Faculty of Pharmacy, University of Coimbra.

2 – Virology laboratory – Hospital and University Center of Coimbra

3 – Clinical laboratory, Faculty of Pharmacy, University of Coimbra

Contacts:

Daniel da Silva Gonçalves

Av. D. Nuno Álvares Pereira, 105 - 3° andar, apartamento 35

4750 - 324 Barcelos (Portugal)

E-mail: leinad_goncalves@hotmail.com

Índex

Table index IV

Acknowledgments V

Abbreviations VII

Resumo: IX

Abstract: XI

1. Introduction 1
   - 1.1 Epidemiology 1
   - 1.2 Structure and genome 1
   - 1.3 HEV genotypes and mode of transmission 2
   - 1.4 Clinical manifestations 3
   - 1.5 Diagnostic 3
   - 1.6 Treatment 4
   - 1.7 Prevention 4
2. Objectives 5
3. Material and methods 7
   - 3.1 Samples 7
     - 3.1.1. Animal samples 7
     - 3.1.2. Wastewater samples 7
   - 3.2. Nucleic acid extraction 7
     - 3.2.1. Extraction preparation 7
     - 3.2.2. Sample preparation 8
     - 3.2.3. Nucleic acid extraction protocol 9
     - 3.2.4. Controls 10
   - 3.3. Viral RNA amplification and detection 10
     - 3.3.1. Taqman® protocol 11
     - 3.2.2. SYBR® Green protocol 11
     - 3.3.3. Real-Time RT-PCR protocol 12
4. Results 13
   - 4.1 Presence of HEV genome in biological samples from wild boars and deers 13
   - 4.2 Prevalence of HEV genome in wastewaters from Portugal 13
5. Discussion and Conclusions 15

References i

Table index

Table 1 - Set of primers used [4] ... 10

Table 2 - TaqMan protocol ... 11

Table 3 - SYBR Green protocol ... 11

Table 4 - Real-Time RT-PCR protocol ... 12

Table 5 - Results from wastewaters ... 13

Acknowledgments

Almost at the end of another goal of my life, it's important to thank to the people who helped me to get this work done and gave me this possibility trusting me this job. First I want to say I'm glad I met these kind of people because I didn't think I would get this level of recognizing from them. I'm talking specifically of my teachers Ana Matos and Ana Donato, because they have always helped me in the conquests I've been making throughout this year. They are like family to me now and all of this makes me feel like I really made the right choice coming into College of Pharmacy from the University of Coimbra.

I want to also leave a compliment to teacher Cristina Luxo for of course all the support in this project and the interest showed in knowing always if everything was going right and discussing the results of the study and tests.

A word to João Vaz due to all his fellowship and enthusiasm in doing the study and support in all that sometimes was needed.

Finally I want to thank to the institution in its all for the possibility that was given to me to do this work and produce scientific knowledge.

Abbreviations

ALT – Alanine transaminase

AVE – Elution buffer

AVL – Lyse buffer

AW – Washing buffer

Ct – Cycle threshold

HCV – Hepatitis C virus

HEV – Hepatitis E vírus

ORF – Open Reading Frame

PBS - Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

RNA – Ribonucleic acid

RT-PCR – Reverse Transcription – Polymerase Chain Reaction

WWTP – Wastewater Treatment Plant

Resumo:

Introdução: O vírus da hepatite E (HEV) pertence ao género *Hepevirus* da família *Hepeviridae*. O HEV é um vírus não envelopado, pequeno e com forma icosaédrica com 30-32 nm de diâmetro e genoma (+) ssRNA. Existem 4 genótipos do vírus (1-4). Os genótipos 1 e 2 estão associados a infecções exclusivamente humanas. Os genótipos 3 e 4 podem infectar suínos e outros mamíferos além dos humanos.

O HEV é responsável por provocar geralmente uma hepatite aguda, sendo mais frequente em países em desenvolvimento. A principal via de transmissão do vírus é a via fecal-oral através da ingestão de águas ou alimentos contaminados. Nas regiões desenvolvidas os genótipos 3 e 4 podem ser responsáveis por focos de infecção devido ao potencial zoonótico.

Objectivo: Avaliar a prevalência da infecção por HEV em javalis e veado de forma a testar a presença do vírus em águas residuais, de forma a avaliar o risco para a saúde pública causado pelo HEV, em Portugal.

Métodos: Trinta amostras de fezes de javalis e veado, 28 amostras de bílis de javali e ainda 30 amostras de águas residuais (15 amostras da entrada da ETAR (Estação de Tratamento de Águas Residuais) e 15 amostras da saída da ETAR), de vários locais do país, foram submetidas a extração do ácido nucleico seguida por amplificação RT-PCR em Tempo Real, para detectar a presença do genoma viral do HEV.

Resultados: Não foi encontrado RNA do HEV em nenhuma amostra de fezes ou de bílis nos animais em estudo.

Nas águas, 2 (13.3%) das 15 amostras colhidas à entrada das ETARs revelaram-se positivas para a presença do genoma do HEV, mas não foi encontrado genoma viral em nenhuma das amostras colhidas à saída da ETAR.

Conclusões: Os resultados do presente estudo sugerem que o HEV ainda não se encontra disseminado pela população de javalis de Portugal. No entanto o HEV está presente no sistema de águas de Portugal e poderá causar epidemias através da ingestão de água contaminada com o vírus. Devemos estar alerta para as infecções causadas por HEV ainda que a maioria delas seja assintomática, uma vez que existe um risco maior para grávidas e doentes imunodeprimidos. Este risco é acrescido por não existir ainda um tratamento eficaz e sem contra-indicações para combater possíveis infecções sintomáticas ou ainda infecções crónicas, quer nos indivíduos saudáveis quer em imunodeprimidos.

Abstract:

Introduction: Hepatitis E virus (HEV) belongs to the *Hepevirus* genus from the *Hepeviridae* family. HEV is a non-enveloped small icosahedral virus with 30-32 nm of diameter and a (+) ssRNA genome. There are four genotypes (1-4) of the virus, genotype 1 and 2 are associated with exclusive infection on humans, while genotype 3 and 4 can also infect pigs and other mammalians. HEV is responsible for a liver disease, generally an acute hepatitis, most frequent in developing countries, where the main way of transmission of HEV is fecal-oral through the ingestion of contaminated water or food. In other regions genotypes 3 and 4 may be causing outbreaks of infection through its zoonotic potential.

Aim: Evaluate the prevalence of HEV infection in wild boars and deer as well as to its presence in wastewaters, in order to evaluate the risk for the public health caused by HEV, in Portugal.

Methods: Thirty samples of wild boar and deer feces, 28 bile samples of wild boars and 30 wastewaters samples (15 samples collected from the influent of the WWTP and 15 samples of the respective effluent of the WWTP) from across country, were submitted to nucleic-acid extraction followed by RT-PCR Real Time amplifications aiming the detection of the viral genome of HEV.

Results: No HEV-RNA was detected in all feces and bile samples from wild animals.

Two (13.3%) out of the 15 influent WWTP samples revealed the presence of HEV-RNA, while the viral genome was not detected in any of the effluent samples.

Conclusion: We find that HEV is not spread across the population of Portuguese wild boars. Nevertheless we acquired that HEV is in fact present in our country which can cause outbreaks by contaminated water ingestion. We must be alert to HEV infections, even if most of them are asymptomatic, there is a high risk for pregnant women and for immunosuppressed population, and until the moment no effective and risk free treatment is available either to a possible chronic infection or even to a clinical symptomatic infection for the general population.

Keywords: Hepatitis E, wild boars, wastewaters, zoonotic infection, viral outbreaks

1. Introduction

1.1 Epidemiology

HEV is responsible for a liver disease, usually an acute hepatitis, most frequent in developing countries.

In these countries the main cases of hepatitis E result from outbreaks associated with ingestion of contaminated water. In developed countries the majority of hepatitis E infections are found to be sporadic and associated with traveling to endemic regions or small outbreaks due to ingestion of contaminated food. More recently some cases were found to be associated with zoonotic spread.

1.2 Structure and genome

HEV belongs to the *Hepevirus* genus from the *Hepeviridae* family. HEV is a small non-enveloped icosahedral virus with 30-32 nm of diameter.[1-3] [2-11] This is a virus with positive single stranded RNA of 7.2kb with three open reading frames (ORF's), who encode both structural and non-structural proteins. (Figure 1) The first open reading frame, located at the 5'end, encodes the majority of enzymes that the virus needs for his replication, such as the RNA-dependent RNA-polymerase, the helicase, the protease and the methyltransferase, which are obtained after the post-translational cleavage that occurs to the polyprotein formed by transcription of ORF1.[1-3, 8-10, 12, 13]

The ORF 2 encodes a structural protein responsible for the constitution of the viral capsid, and is located at the 3' end of the genome.

Finally the ORF 3 overlaps the other two ORF's and encodes a phosphoprotein, which seems to be helpful in the virus replication and assembly plus cytoskeleton synthesis, but its function it's not yet fully known.

It is also known that the virus as a hipervariabe region on the ORF 1 that is responsible for the four known different genotypes.[2, 3, 9-12]

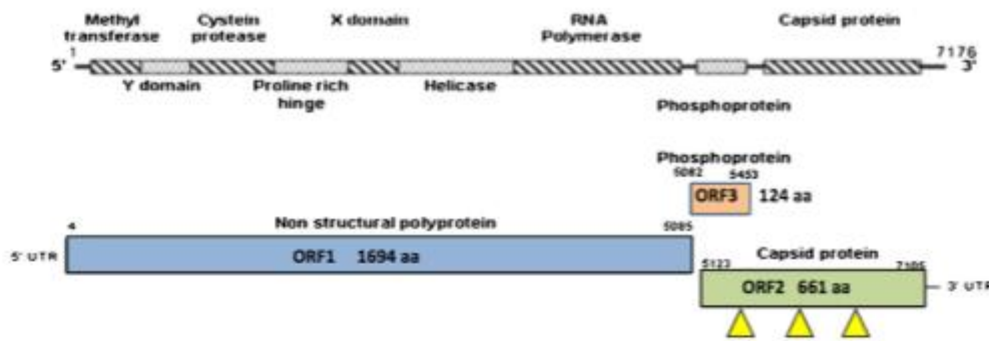

Figure 1 - HEV genome scheme(11)

1.3 HEV genotypes and mode of transmission

There are four genotypes of the hepatitis E virus (1-4). Genotypes 1 and 2 are exclusevely associated with infection on humans. Genotypes 3 and 4 are thought to infect pigs and other mammalians besides humans.[2, 11] Its these genotypes 3 and 4 that can lead us to suspect that the virus has a zoonotic potential.[4-7, 14-18] This suspicious results from several reports of sporadic cases of HEV infections on people living in countries without epidemiologic evidence of HEV infections and that hasn't left their home country for a long period of time. The occurrence of these sporadic cases points to a possible different mode of transmission such as some kind of contact of those people with animals infected with HEV, for example.[6, 16-23] These kind of situations have been associated with genotypes 3 and 4. It's known that these specific genotypes infects wild boars and others wild animals as well as domestic pigs that are used for human consumption.[1, 14, 15, 17, 19] It's likely that the raw or undercooked meat can host the virus or even an occupational infection can occur.[7, 20, 23] If the meat isn't cooked properly and for the necessary time at a minimum temperature that destroy the virus it can be the vehicle for the infection, because being a naked virus as is known gives a greater resistance to these kind of virus and so they can still be viable even throw harsh conditions.[7, 15, 20, 23] An occupational infection may also occur in people that work at slaughterhouses due to the close contact with the animals.[20, 23, 24] The evaluation of the zoonotic nature of HEV infection is of extreme importance, as well as the estimation of the number of animals that it affects as well as the possibility of these animals to infect humans. By this manner it could be more efficient to prevent the population of being infected and create new groups of risk to this infection, such as hunters, pregnant women or even immunosuppressed people, for example.[2, 8, 14, 17, 18, 22, 23, 25]

Another cause for these infections is the consumption of contaminated water, which can reach the natural courses of water and expose the population to the HEV.[7, 20, 23] Furthermore fruits or vegetables that may have been in contact with this water may also be

contaminated and possibly transmit the virus.[7, 23] So by this whole scheme we see that the infection with HEV may represent a serious problem of public health.[1, 8, 13, 17, 18, 21-23]

Previous studies revealed that HEV can become a chronic infection in immunosuppressed patients, so it's highly important to evaluate all the possible ways of its transmission specially to this specific population, which as we know is increasing in our country, much like in the rest of the world.[26]

1.4 Clinical manifestations

Normally the HEV infection is self-limited as an acute hepatitis with a period of incubation of 40 days in average.[2, 3, 10, 12, 17, 27] It manifests as an icteric hepatitis normally allied with fever, abdominal pain, vomiting, dark urine, hepatomegaly and transaminases can also be high.[2, 3, 12, 17, 27]

Thirty to 50% of the infections are asymptomatic. The frequency and severity of clinical manifestations depend on the age of the affected individual, because different ages have different types of immune response and it's known that the consequences of the HEV infection depend highly on the immune system of the infected individual, so clinical manifestations are higher in early to mid-adulthood. HEV seems to have a special harshness in pregnant women with a higher risk of fulminant hepatitis in this population.[3, 27]

1.5 Diagnostic

The initial response by immune system to HEV infection is the production of specific IgM type antibodies at around 2 to 3 weeks after exposure to the virus.[17, 28] Meanwhile the ALT (aspartate aminotransferase) reaches its peak by the same time as the first antibodies appear and HEV-RNA can also be detected in stool and serum samples from this period until 2 to 3 months after exposure, but its presence will disappear first in serum and later on stool samples too.[12, 28] Shortly after the IgM, the IgG specific antibodies will be produced against the virus and these type of antibodies will be maintained for a much longer period of time than the first ones. Nevertheless, it's shown that these antibodies will also decrease through time as well, so we can assume that maybe they aren't lifetime protectors against possible re-exposure to the virus in the future.[28] (Figure 2)

The diagnostic of the infection is mostly based on serology with the search of anti-HEV IgM and anti-HEV IgG, and like other types of hepatitis a trace of the infection state can be made with these markers.

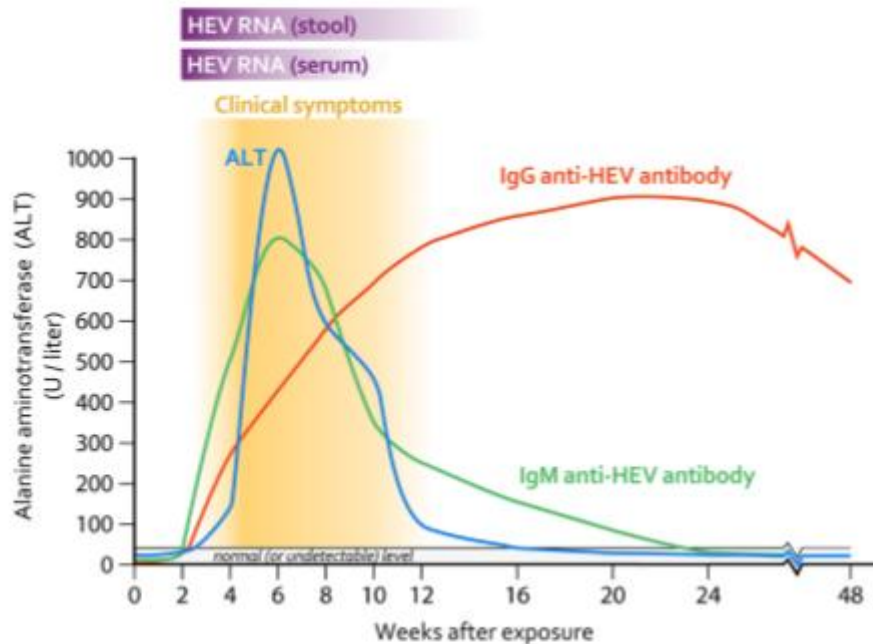

Figure 2- Clinical manifestations and markers of HEV infection(28)

1.6 Treatment

If HEV can become a chronic infection it is highly important that a treatment to this infection can be able to prevent or treat it.[2, 17, 22, 26]

The treatment that has been tried and for now is showing some results is the same as used for treatment of HCV, by using the ribavirin and sometimes the association of it with the pegylated α-interferon.[2, 29] But this treatment has several problems and many side effects, such as influenza-like symptoms, neuropsychiatric effects and severe anemia. By this, it can't be used to treat every type of infection, such as mild infections or more important the infection of pregnant women.[29]

1.7 Prevention

The prevention of infection by HEV passes through the ingestion of drinkable water mainly in endemic regions, implementation of correct habits of hygiene and the proper cooking of all meat and vegetables.[12]

A vaccine exists but it's not commercialized, and for now it's only being used on Asia because of the higher prevalence of the infection in that region.

2. Objectives

The aim of this study is to search for the prevalence of HEV infection in wild animals such as wild boars and test the presence of the virus himself in water used by humans in their normal daily routines and own consumption, in order to evaluate the risk for the public health caused by HEV.

For that purpose, the presence of HEV genome was evaluated in stool and bile samples of wild boars and deer. To further explain and comprehend the levels of HEV circulating in the Portuguese community we also tested samples of wastewater treatment stations (WWTP) around the country, evaluating both influents and effluents of the station. By doing this study we can comprehend the spread of the virus in our country, both in wild animals and in the waters circulating and served to the population. Further, we can also ascertain if the treatment used in the water stations is suitable to fully destroy the virus in case it is present in the water that reaches the station.

3. Material and methods

3.1 Samples

3.1.1. Animal samples

Stool and bile samples were obtained from a group of 29 wild boars and 1 deer, from the regions of Santarém and Portalegre.

The samples were collected by a veterinarian in the proper amount needed and treated as dangerous because they could be contaminated with HEV and with minimal environmental contamination. Bile was extracted from the bile ducts and feces were removed directly from the bowls of the boars. Before extraction all samples were stored at -20°C until processed, which keeps the viral RNA stable and with no risk of getting digested or fragmented from bacteria or environmental factors.

A total of 30 stool samples and 28 bile samples were available for the study.

3.1.2. Wastewater samples

Wastewater samples from the influent and effluents of 15 WWTP of different regions of Portugal, were collected into sterile containers and stored at -20°C until process.

A total of 30 wastewater samples (15 from the influents and 15 from the effluents) was available for the study.

3.2. Nucleic acid extraction

In order to obtain the virus RNA the extraction kit QIAamp® Viral RNA Mini Kit (50) from QIAGEN, was used for all samples. Besides enabling the purification of RNA, this kit is also suitable to eliminate the major causes of interference in the PCR reaction.

3.2.1. Extraction preparation

Before start the nucleic acid extraction protocol, it is need to:

- Balance the samples to room temperature
- Make sure that buffers AW1 and AW2 were properly prepared
- Prepare the mix AVL buffer/ Carrier RNA
- Add 16,8µl of Carrier RNA previously reconstituted and preserved at -20°C to 1,68ml of AVL buffer (3 extractions)
- This mix is stable for 48h at 2-8°C

- If the AVL buffer presents some kind of precipitate it must be dissolved by warming up the buffer to 37ºC, and then let it cool to room temperature
- Prepare a support with the following tubes:
- 2 x **n** eppendorf tubes of 2ml, for lysis phase
- **n** extraction columns
- 4 x **n** collecting tubes of 2ml
- **n** eppendorf tubes of 1,5ml, for the final elution of the viral RNA in which **n** is the number of samples processed
- Correctly identify both tubes and columns matching them with the samples, putting also the date in the tube that will contain the eluate of viral RNA
- For every round of extractions a negative control must be used, which should be made with sterile water instead of sample.

3.2.2. Sample preparation

3.2.2.1. Bile samples

Bile samples were ready for nucleic acid extraction after being collected, so they could be used directly without any previous preparation procedure.

3.2.2.2. Stool samples

Two hundred miligrams of stool samples were suspended in 1.8ml of PBS. After mixing, a centrifugation was performed, for 15min at 3000g, and the supernatant was collected to an Eppendorf tube of 1.5ml, and stored at -20ºC until nucleic acid extraction.

3.2.2.3. Wastewater samples

Wastewater samples were submitted to a ultracentrifugation protocol as described below, in order to concentrate the present viral particles.

1. Properly homogenize the water
2. Pipette 25ml of water for each one of the 3 specific tubes of the ultracentrifuge
3. Balance the tubes, with help from a balance
4. Put the tubes on the rotor, close it, and put it in the ultracentrifuge
5. Centrifuge for 90min at 41000 rpm and at room temperature
6. In the end collect the supernatant and preserve it at -20ºC
7. Release the pellets on 500µl of supernatant and transfer it to a glass tube, correctly sterilized
8. Add about 500µl of chloroform
9. Mix in the vortex

10. Centrifuge at 1500 rpm for 10 min
11. Collect the liquid phase (superior one) to an Eppendorf tube of 1,5ml and conserve it at -20ºC until analysis

3.2.3. Nucleic acid extraction protocol

A volume of 140µl of sample prepared as described in previous sections were submited to the following nucleid acid extraction protocol.

Protocol:

1. Pipette 560µl of the mix AVL buffer/ carrier RNA for each *Eppendorf* tube of 2ml
2. Add 140µl of the sample
3. Mix on vortex for 15 seconds, immediately after addiction on each tube
4. Incubate at room temperature (15-25ºC) for 10min
5. Do a brief centrifugation of the tubes, to remove some drops that may be in the inside walls of the tubes or on the cap
6. Add 560µl of ethanol (96-100%)
7. Mix on vortex for 15 seconds, immediately after addiction on each tube
8. Carefully apply 630µl of the previous mix into the column, properly labeled and with a collecting tube on it. It shouldn't stay any air bubble between the filter of the column and the liquid.
9. Centrifuge at 8000 rpm for 1 min at room temperature. If some part of the solution didn't get filtered it must be centrifuged, at a higher speed, until all solution passes through the membrane
10. Put the column on a new collecting tube and reject the previous one. Open the column gently.
11. Carefully apply 630µl of the previous mix (from step 7) into the column, properly labeled and with a collecting tube on it. It shouldn't stay any air bubble between the filter of the column and the liquid.
12. Centrifuge at 8000 rpm for 1 min at room temperature. If some part of the solution didn't get filtered it must be centrifuged, at a higher speed, until all solution passes through the membrane
13. Put the column on a new collecting tube and reject the previous one. Open the column gently.
14. Add 500µl of AW1 buffer. Close the column.
15. Centrifuge at 8000 rpm for 1 min at room temperature

16. Put the column on a new collecting tube and reject the previous one. Open the column gently.
17. Add 500µl of AW2 buffer. Close the column
18. Centrifuge at max speed (14000 rpm) for 3 min at room temperature
19. Put the column on a new collecting tube and reject the previous one. Open the column gently.
20. Centrifuge at max speed for 1min
21. Put the column on a new collecting tube and reject the previous one. Open the column gently.
22. Add 60µl of AVE buffer, balanced at room temperature. Close the column
23. Incubate at room temperature for 1min
24. Centrifuge at 8000 rpm for 1 min at room temperature
25. The solution in the bottom of the collecting tube contains the viral RNA (stable for 1 year if kept between -20 and -80°C).

3.2.4. Controls

Negative and positive controls were included in all experiment.

HEV RNA was mixed with each of the different type of studied samples. In order to assure that the extraction procedure worked out as expected.

Negative controls consisting of RNAse free water instead of normal sample, were also included in each experiment, in order to assure the absence of contamination problems.

3.3. Viral RNA amplification and detection

Extracted nucleic acid obtained from all the samples were submitted to a RT-qPCR in order to evaluate the presence of HEV genome. A set of 2 primers specific for the ORF2 region of HEV genome were used. (Table 1)[4]

Table 1 - Set of primers used [4]

		Position
HEV Forward primer	CCGACAGAATTRATTTCGTCGGC	6296 - 6318
HEV Reverse primer	ATACCCTTRTCYTGCTGIGCRTTCTC	6420 - 6395
HEV-Probe	FAM-CTCGCCATTGGCTGAGAC-MGB-NFQ	6367 - 6350

Two amplification protocols were used.

3.3.1. Taqman® protocol

This amplification protocol used a TaqMan chemistry for detection of the amplified product. For that purpose a TaqMan probe specific for the ORF2 region of HEV genome was used. (Table 1)

Amplification reaction was carried out in a total volume of 25µl, containing the reagents described in Table 2.

Table 2 - TaqMan protocol

Superscript® III Platinum® One-Step qRT-PCR System kit
300µM of each primer
60µM of HEV probe
12.5µl reaction mix *
5µl of RNA
RNAase Free water
0.8µl of Superscript™ III RT/Platinum® Taq emzyme

* the reaction mix already contains 0.4mM of each dNTP and 2.4mM of $MgSO_4$.

3.2.2. SYBR® Green protocol

This amplification protocol used SYBR Green chemistry for detection of the amplified product.

The amplification was carried out in a total volume of 25µl, containing the reagents indicated in Table 3:

Table 3 - SYBR Green protocol

iScript™ One-Step RT-PCR Kit With SYBR® Green kit
300µM of each primer
12.5µl of 2x reaction buffer *
5µl of RNA
RNAase Free water

* the reaction buffer already contains 0.4mM of each dNTP, iTaq DNA polymerase and 20nM of SYBR Green.

3.3.3. Real-Time RT-PCR protocol

Amplification of nucleic acid and detection of amplified products were carried on *BIORAD CFX-96* ® thermal cycler equipment. Real-Time RT-PCR reaction was performed under the conditions below (Table 4):

Table 4 - Real-Time RT-PCR protocol

	SYBR® Green		*TaqMan*	
Hold-RT	50°C	10min	50°C	30min
Denature	95°C	5min	95°C	2min
Cycling (45cycles)	95°C	10sec	95°C	15sec
	60°C	30sec	60°C	60sec

4. Results

4.1 Presence of HEV genome in biological samples from wild boars and deers

Thirty stool samples and 28 bile samples collected from 29 wild boars and 1 deer were examined for the presence of HEV genome through 2 different amplification protocols.

No HEV genome was detected in any of the evaluated stool samples.

In respect to the bile samples, 23 out of the 28 studied samples rendered an undetectable result, and the other 5 samples revealed a suspicious result by the SYBR Green protocol. Nevertheless, the analysis of these 5 samples through the TaqMan protocol confirmed the inexistence of detectable HEV-RNA.

4.2 Prevalence of HEV genome in wastewaters from Portugal

Thirty wastewater samples from different regions of Portugal were evaluated for the presence of HEV genome through the TaqMan amplification protocol described above.

The obtained results are shown in Table 5.

Table 5 - Results from wastewaters

Sample type	Number of samples	
	Tested	With HEV genome (%)
Influent (WWTP)	15	2 (13.3%)
Effluent (WWTP)	15	0 (0%)
Total	30	2 (6.67%)

5. Discussion and Conclusions

We evaluate Portuguese wild animals and wastewaters for the presence of HEV, in order to clarify the possible danger to the public health caused by the hepatitis E virus.
From the results obtained with the two amplification protocols, it was evident that the use of Real Time RT-PCR *TaqMan* protocol is preferable to the SYBR Green protocol, once nonspecific amplification was observed with the last one in some samples. The evaluations of these samples through the TaqMan protocol render undetectable results. These results may be associated with the low number of RNA copies in samples or it can also be due to unspecific amplification with SYBR Green, as this protocol doesn't have a specific probe to the genome and binds to all double stranded chains that may appear in the amplification reaction.
All wild boars and deer samples were found negative to HEV genome, so we may be leading to think that HEV is not present in great quantity in the wild animals of our country. In a similar Spanish study, HEV-RNA was found in 37.7% of the animals tested, being detected in 19.1% of the bile samples and in 12.3% of feces samples. [14] Another similar study in Portugal showed a prevalence of HEV up to 30% in domestic pigs. [5] There are also published data about HEV autochthonous infections, in Portugal and India, showing that the virus has indeed a zoonotic potential and the source of the infections must be the environment or animals like wild boars or domestic pigs. [4, 6] The fact we didn't find any positive sample could be related with the use of different primers and different conditions of amplification and extraction. We have also to consider the fact that we used a smaller number of samples (the previous study was performed on 69 animals).
Seroepidemiological studies demonstrated the presence of HEV specific antibodies in 51% of specific populations, like swine farmers, while the seroprevalence for the general population was of 25% only. [1, 15] Another study, revealed a seroprevalence of 31% in forest workers.[24]
It is highly important to understand if these populations are at high risk of infection due to its higher level of exposure to HEV, through the contact with infected animals.
It would be important to fully understand the spread of HEV in Portugal. For the healthy population, from around the different continents, a major study was made to evaluate the prevalence of antibodies against HEV, and the data found showed a lower prevalence of antibodies on the samples tested in comparison to the high exposure population samples, like farmers and forest workers.[25] But when the study was performed in a greater number of samples, the percentage of antibodies found also raised proportionally, to levels of 77% in

the Asia region and 27% in Europe. [25] A study with blood donors on Europe showed a sero prevalence of 6.8%. [13] Since the majority of these studies were made in developed countries we can discard the main transmission way of HEV. By this we have to think that the percentages found in the different studies can't be all due to exclusively human HEV genotypes (1-2), but more likely the transmission and contact with HEV should be the zoonotic way with genotypes 3 and 4.

During our study, two water samples were found positive, which lead us to think that the contamination of those waters may be due to feces from infected animals or even humans. Anyway, it would be important to know the origin of this contamination. An hypothesis chance is that the contamination of waters comes from domestic pigs raised in farms or for personal consumption, once they are related to wild boars and as published before, pigs represent one of the most frequently infected animals by HEV.

To get the idea of the reason those waters were contaminated we need to study animal populations from the regions were the samples of wastewaters were found positive and further analyze the nucleotide sequence of HEV-RNA found on pigs as well as those found in the water samples. If the genotype is the same we can assume that the soil contamination from pigs feces leads to consequent water contamination. If none or very few of the pigs is found infected we need to found if the contamination of waters is made by human contamination.

Furthermore, it would also be interesting to evaluate the HEV infection in Portuguese individuals through a seroprevalence study, doing the screening of specific groups of population for either IgG or IgM against HEV, such as hunters, farmers, pregnant women and immunosuppressed patients since these are the high risk groups because of the exposure in one case and for the consequences of the infection in the other.

Another problem raised from our study is the fact that if water samples were found positive we know that HEV is circulating in the environment. Both positive influent samples for HEV-RNA were found negative on the effluent samples, so we could say that the treatment process is working in the right way for the elimination of the virus from water used by humans.

In conclusion we acquired that HEV is present in our country with the risk to cause outbreaks by contaminated water ingestion and since we can’t discard the HEV infection just due to the majority of the cases be asymptomatic, there is a high potential for HEV to become a serious public health problem.

References

1. Freshwater DA. Hepatitis E: the forgotten virus. Journal of the Royal Army Medical Corps. 2013;159(3):167-8.

2. Dalton HR, Pas SD, Madden RG, van der Eijk AA. Hepatitis e virus: current concepts and future perspectives. Current infectious disease reports. 2014;16(4):399.

3. Kamar N, Dalton HR, Abravanel F, Izopet J. Hepatitis E virus infection. Clinical microbiology reviews. 2014;27(1):116-38.

4. Rolfe KJ, Curran MD, Mangrolia N, Gelson W, Alexander GJ, L'Estrange M, et al. First case of genotype 4 human hepatitis E virus infection acquired in India. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology. 2010;48(1):58-61.

5. Berto A, Backer JA, Mesquita JR, Nascimento MS, Banks M, Martelli F, et al. Prevalence and transmission of hepatitis E virus in domestic swine populations in different European countries. BMC research notes. 2012;5:190.

6. Duque V, Ventura C, Seixas D, da Cunha S, Melico-Silvestre A. First report of acute autochthonous hepatitis E in Portugal. Journal of infection in developing countries. 2012;6(2):201-3.

7. Yugo DM, Meng XJ. Hepatitis E virus: foodborne, waterborne and zoonotic transmission. International journal of environmental research and public health. 2013;10(10):4507-33.

8. Aggarwal R. Hepatitis E: Historical, contemporary and future perspectives. Journal of gastroenterology and hepatology. 2011;26 Suppl 1:72-82.

9. Aggarwal R, Jameel S. Hepatitis E. Hepatology. 2011;54(6):2218-26.

10. Abravanel F, Lhomme S, Dubois M, Peron JM, Alric L, Kamar N, et al. Hepatitis E virus. Medecine et maladies infectieuses. 2013;43(7):263-70.

11. Perez-Gracia MT, Mateos Lindemann ML, Caridad Montalvo Villalba M. Hepatitis E: current status. Reviews in medical virology. 2013;23(6):384-98.

12. Mushahwar IK. Hepatitis E virus: molecular virology, clinical features, diagnosis, transmission, epidemiology, and prevention. Journal of medical virology. 2008;80(4):646-58.

13. Juhl D, Baylis SA, Blumel J, Gorg S, Hennig H. Seroprevalence and incidence of hepatitis E virus infection in German blood donors. Transfusion. 2014;54(1):49-56.

14. de Deus N, Seminati C, Pina S, Mateu E, Martin M, Segales J. Detection of hepatitis E virus in liver, mesenteric lymph node, serum, bile and faeces of naturally infected pigs affected by different pathological conditions. Veterinary microbiology. 2007;119(2-4):105-14.

15. Meng XJ. From barnyard to food table: the omnipresence of hepatitis E virus and risk for zoonotic infection and food safety. Virus research. 2011;161(1):23-30.

16. Izopet J, Dubois M, Bertagnoli S, Lhomme S, Marchandeau S, Boucher S, et al. Hepatitis E virus strains in rabbits and evidence of a closely related strain in humans, France. Emerging infectious diseases. 2012;18(8):1274-81.

17. Song YJ, Park WJ, Park BJ, Lee JB, Park SY, Song CS, et al. Hepatitis E virus infections in humans and animals. Clinical and experimental vaccine research. 2014;3(1):29-36.

18. Echevarria JM, Fogeda M, Avellon A. [Epidemiology of hepatitis E virus infection in Spain.]. Enfermedades infecciosas y microbiologia clinica. 2014.

19. Ruggeri FM, Di Bartolo I, Ponterio E, Angeloni G, Trevisani M, Ostanello F. Zoonotic transmission of hepatitis E virus in industrialized countries. The new microbiologica. 2013;36(4):331-44.

20. Arends JE, Ghisetti V, Irving W, Dalton HR, Izopet J, Hoepelman AI, et al. Hepatitis E: An emerging infection in high income countries. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology. 2014;59(2):81-8.

21. Dalton HR. Hepatitis e: the 'new kid on the block' or an old friend? Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie. 2014;41(1):6-9.

22. Dreier J, Juhl D. Autochthonous Hepatitis E Virus Infections: A New Transfusion-Associated Risk? Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie. 2014;41(1):29-39.

23. Van der Poel WH. Food and environmental routes of Hepatitis E virus transmission. Current opinion in virology. 2014;4:91-6.

24. Carpentier A, Chaussade H, Rigaud E, Rodriguez J, Berthault C, Boue F, et al. High hepatitis E virus seroprevalence in forestry workers and in wild boars in France. Journal of clinical microbiology. 2012;50(9):2888-93.

25. Echevarria JM. Light and Darkness: Prevalence of Hepatitis E Virus Infection among the General Population. Scientifica. 2014;2014:481016.

26. Fujiwara S, Yokokawa Y, Morino K, Hayasaka K, Kawabata M, Shimizu T. Chronic hepatitis E: a review of the literature. Journal of viral hepatitis. 2014;21(2):78-89.

27. Aggarwal R. Clinical presentation of hepatitis E. Virus research. 2011;161(1):15-22.

28. Krain LJ, Nelson KE, Labrique AB. Host immune status and response to hepatitis E virus infection. Clinical microbiology reviews. 2014;27(1):139-65.

29. Debing Y, Neyts J. Antiviral strategies for hepatitis E virus. Antiviral research. 2014;102:106-18.