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(54) Title: RT-PCR TEST FOR THE DETECTION OF AVIAN HEPATITIS E VIRUS

(57) Abstract: The present invention relates to methods for the detection of the presence of avian hepatitis E virus in a sample by means of a specific primer set and optionally a specific probe and to diagnostic test kits for the detection of the presence of avian hepatitis E virus in a sample that comprise such a specific primer set and optionally a specific probe.



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RT-PCR test for the detection of avian Hepatitis E Virus

The present invention relates to methods for the detection of the presence of avian hepatitis E virus in a sample and to diagnostic test kits for the detection of the presence of avian hepatitis E virus in a sample.

Avian Hepatitis E Virus (avian HEV) is the causative agent of big liver and spleen disease (BLSD) and hepatitis-splenomegaly syndrome (HSS) which were reported in Australia, Northern America and Europe (24). It was found to be enzootic in chicken flocks in the USA (15). Also in apparently healthy chicken flocks a high prevalence of antibodies was detected and avian HEV was isolated (30). In Europe wide distribution of avian HEV was reported (2, 21, 22, 25, 28, 32).

Avian HEV has not been isolated from other species than chicken, so far. Experimentally it was shown that it is able to cross the species barrier and infect turkeys (31), but not rhesus monkeys (17). Together with human and swine HEV, avian HEV belongs to the genus *Hepevirus* within the new family *Hepeviridae* (7). Phylogenetic analysis revealed that avian HEV forms a separate genus, itself consisting of at least three different genotypes and a geographic distribution pattern was observed (2, 21). Recently, HEVs detected in Norway rats were genetically identified (18). Furthermore, HEVs have been reported in several other animal species (23).

Due to problems in efficiently propagating Hepatitis E virus in cell culture, the diagnosis is currently based on antibody tests (ELISA, AGID) and conventional reverse transcription-polymerase chain reaction (RT-PCR) (23). Purified viral antigen was used in the first ELISA to detect avian HEV specific antibodies in serum samples (32). Later an ELISA was developed on the basis of a truncated capsid protein of avian HEV, of which its cross-reactivity with human and swine HEV was shown (11). Based on this ELISA several studies about seroprevalence of avian HEV were carried out (15, 28, 30). A few RT-PCRs are described for avian HEV (15, 27, 30, 31) but real-time RT-PCR (SYBR-Green or TaqMan) methods exist only for human and swine HEV (1, 8, 10, 19, 20, 26). Not all of them are quantitative and only one (1) uses *in vitro* transcribed RNA as a standard for quantification. (SYBR-Green based RT-PCR methods have been described by Mackay, I.M. et al. (5). TaqMan based RT-PCR methods are a development by Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404, USA).

The genomic organization of avian HEV is similar to human and swine HEV, both consisting of three open reading frames (ORFs) and two short non coding regions (NCRs). The length of avian HEV genome (6.6 kb) is about 600 bp shorter than human and swine HEV genome and they share only approximately 50 to 60 % sequence identity (2, 15, 17, 27). Therefore the molecular methods for detection of mammalian HEV are not suited for avian HEV.

Especially for RNA viruses that have highly variable genomes, the choice of appropriate primer and probe binding sites is crucial.

RT-PCR methods for detection of avian HEV used so far are of unknown specificity for samples of different geographic origin (23). Many RT-PCRs for detection of avian HEV RNA described in literature use primers especially designed to anneal to the sequence of the isolate that was used in the same study (3, 4, 11, 12, 16, 17, 31). In Australia, a two-step RT-PCR was developed, that could detect RNA in liver samples from BLSD affected flocks (27). Most of the RT-PCR methods for detection of avian HEV RNA are based on degenerate primers, binding within ORF1 (15) or on a nested approach applying degenerate primers binding within ORF1 and ORF2, respectively (30). These primers have e.g. been developed for RT-PCR detection of isolates from Northern America. Additionally, primers have been developed for RT-PCR detection of European and Australian avian HEV (2, 21).

A disadvantage of the primers used is, that they have a high specificity for avian HEV strains of the same geographical origin, but therefore they may possibly not react with avian HEV strains of a different geographical origin. As a consequence, the presence of some avian HEV strains in chicken flocks as well as in biological material may remain unnoticed.

It is clear that reliable diagnostic tools for the detection of avian HEV in chicken flocks are essential, if only to detect the presence or absence of avian HEV in an animal. Such a tool would also be essential to monitor the geographical spreading of avian HEV. This could i.a. reveal the presence or absence of avian HEV strains from a certain geographical origin in chickens on a different geographical location.

There is also a need for reliable diagnostic tools in the field of vaccine production. Many of the avian virus vaccines (and not only avian vaccines) are grown on eggs or in chicken cell cultures. So it is important to check for the absence of avian HEV in these eggs or in chicken cell cultures.

Thus there is a clear need for reliable methods and diagnostic tools that are able to detect avian HEV strains irrespective of the geographical origin of those HEV strains.

An even greater need exists for quantitative methods and diagnostic tools that are able to give an indication of not only the presence but also the amount of HEV in various types of sample material. This would i.a. greatly facilitate studies about the pathology of avian HEV.

It is an objective of the present invention to provide such methods and diagnostic tools.

Surprisingly now a specific primer set was found that, if desired in combination with a specific probe, is capable of detecting avian HEV strains irrespective of the geographical origin of those HEV strains.

Detection of HEV in a sample can be done i.a. by performing the following method steps:

a) performing a reverse transcriptase polymerase chain reaction (RT-PCR) of said sample using a primer set comprising a primer that comprises a stretch of at least 16 consecutive nucleotides from

an oligonucleotide BLSV-HEVf having a sequence as depicted in SEQ ID NO.: 5 and a primer that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVr having a sequence as depicted in SEQ ID NO.: 6 and

b) examining the RT-PCR amplification result of step (a)

5

The term “primer” is used to describe an oligonucleotide that is capable to recognise, and to bind to, a complementary polynucleotide and to act as an initiation point for nucleic acid synthesis or replication along a complementary strand.

10 The term “probe” refers to an oligonucleotide that is complementary to a polynucleotide of interest, and capable of forming, under hybridisation conditions, a duplex structure with that polynucleotide. A probe doesn’t need to be fully complementary to the polynucleotide of interest, as long as the hybridisation conditions are such that annealing can take place in spite of the incomplete complementarities.

15 The word “probe” basically refers to a primer that additionally carries a specific marker. Such a marker can i.a. be a fluorophore, such as the fluorophores used in e.g. the TaqMan probes (*vide infra*).

The term “oligonucleotide” is used herein to describe a short polymer of nucleic acids. Such short polymer would usually have a length of between 10 and 100 nucleic acids.

20 The term “hybridisation conditions” relates to conditions that allow the primer or probe to anneal to the polynucleotide of interest. These conditions depend on the temperature and the ionic strength of the solution in which hybridisation is to take place. Hybridization reactions and conditions are well-known in the art and are i.a. described in the standard laboratory manual by Maniatis/Sambrook (34).

25 PCR-techniques are equally well-known in the art and they are extensively described in standard laboratory manuals such as; Real-Time PCR: Current Technology and Applications (35), in PCR primers, a laboratory manual (36), and in Maniatis/Sambrook (34).

30 The primers and the probe according to the invention can inter alia be used for the detection of avian HEV in an animal or a sample of that animal.

35 The term “sample” is used herein to refer to any biological material that is suspected of containing avian HEV. The biological material can i.a. be tissue such as chicken liver, spleen, bone or muscle tissue, but it can also be a body fluid such as e.g. blood, urine, faecal material, amniotic fluid. The material can also be a cloacal, oral or nasal cavity swab or e.g. disrupted cells.

It goes without saying that the material to be tested can also be of non-avian origin. It may well be that non-avian species are suspected of carrying avian HEV, or are tested in order to exclude that they carry avian HEV.

A reverse transcriptase polymerase chain reaction, further referred to as RT-PCR comprises two reaction steps. In a first step, one of the primers of the primer set is allowed to bind to HEV-RNA, and this complex forms the starting point for the synthesis of a cDNA strand of the RNA strand by the enzyme reverse transcriptase (an RNA-dependent DNA polymerase) in the presence of the four DNA building blocks A, T, G and C.

In a second step, the thus-formed RNA-DNA hybrid is heated in order to denature the hybrid, followed by cooling in order to allow the other primer of the primer set to bind to the cDNA strand. This other primer then functions as the starting point for the synthesis of the second DNA strain by a DNA polymerase, again in the presence of the DNA building blocks.

Depending on the amount of HEV-RNA in the sample (provided that it is present), several PCR-cycles will have to be made before there is sufficient material to be detected. An average of between 30 and 50 cycles would not be unusual. The skilled artisan would be able, on the basis of the sequences of the primers and the probe, to determine the optimal temperature conditions for the various steps of the PCR-cycle using e.g. the formulae given in text books such as (34), (35) and (36). (*vide supra*).

The oligonucleotide BLSF-HEVf has the nucleotide sequence AAT GTG CTG CGG GGT GTC AA. Thus, examples of a stretch of at least 16 consecutive nucleotides from BLSV-HEVf therefore are AAT GTG CTG CGG GGT G, T GTG CTG CGG GGT GTC and AAT GTG CTG CGG GGT GTC A.

The term “a primer comprising a stretch of at least 16 consecutive nucleotides from an oligonucleotide having a sequence as depicted in SEQ ID NO.:” means that the primer should at least have the length of a stretch of at least 16 consecutive nucleotides from the oligonucleotide as depicted in that SEQ ID NO. Merely as an example: the primer BLSV-HEVf has the sequence aatgtgctgc ggggtgtcaa as depicted in SEQ ID NO.: 5. Thus, a “primer comprising a stretch of at least 16 consecutive nucleotides from an oligonucleotide having a sequence as depicted in SEQ ID NO.:” should at least consist of a stretch of at least 16 consecutive nucleotides from the nucleotides aatgtgctgc ggggtgtcaa in that order. It could however be a longer primer that e.g. comprises the nucleotides aatgtgctgc ggggtgtcaa, and has one or more additional nucleotides at the 5'-end and/or the 3'-end.

The same is true for the probe (although it is clear that the probe should not have a length at which the quenching molecule is no longer quenching the fluorophore; *vide infra*). The oligonucleotide of the probe as depicted in SEQ ID NO.: 8 has a length of 22 nucleic acids, but again, the oligonucleotide of a probe according to the invention should have a minimal length of at least 16 consecutive nucleotides from an oligonucleotide having a sequence as depicted in SEQ ID NO.: 8.

If a primer (or probe) is chosen that has additional nucleotides at the 5'-end and/or the 3'-end, such nucleotides may or may not be complementary to the 3'- and/or 5'-flanking regions of the complementary strand to which the primer binds. In some cases, the temperatures of the various RT-PCR cycles should possibly be adapted to the increased length of the primer and to the fact that one or more of the additional nucleotides are complementary. And again; the skilled artisan would be able, on the basis of the sequences

of the primers and the probe, to determine the optimal temperature conditions for the various steps of the PCR-cycle using e.g. the formulae given in PCR text books referred to herein (*vide supra*).

Primers comprising a stretch of 17, 18, 19 or even 20 consecutive nucleotides from an oligonucleotide having a sequence as depicted in SEQ ID NO.: 5 or 6 are preferred in this order of preference, since they anneal even more selectively to avian HEV-sequences.

Primers or probes comprising a stretch of 17, 18, 19, 20, 21 or even 22 consecutive nucleotides from an oligonucleotide having a sequence as depicted in SEQ ID NO.: 8 are preferred in this order of preference, since they also anneal even more selectively to avian HEV-sequences.

In principle, after step a) of the method of the present invention, there are now different ways to perform step b). The PCR step results in a PCR product of which the length and amount can be examined e.g. by means of conventional agarose or polyacrylamide gel electrophoresis. In case there is avian HEV RNA present in the sample, the primer pair would anneal and therefore, after step a), a PCR product of the expected length would be detected on gel. If there is no avian HEV RNA present in the sample, the primer pair, or at least one of the primers, would fail to anneal and therefore, no PCR product of the expected length would be detected.

An alternative to the time-consuming analysis of the potential PCR-product by means of conventional agarose or polyacrylamide gel electrophoresis is the use of the SYBR Green system (*vide supra*). SYBR Green is a dye that intercalates with double-stranded DNA. This intercalation causes SYBR Green to fluoresce. Therefore, if the RT-PCR reaction is done in the presence of SYBR Green, each new cDNA copy would pick up an amount of SYBR Green and cause it to fluoresce. A qPCR machine can detect the fluorescence and software can calculate Ct values from the intensity of the fluorescence. This allows for a direct quantification of the amount of cDNA made. (The use of SYBR Green however does not allow for the presence of an internal control that indicates if the reaction steps proceeded as expected).

As can be seen from table 4, a conventional RT-PCR with either the primers HelicaseF/HelicaseR or Forw1_C-BLSV/Rev1_C-BLSV gives a PCR-product in only 13 out of 16, or 9 out of 16 avian HEV field-samples. This shows that these probes are not suitable for the detection of all avian HEV-viruses regardless their geographical origin.

It follows also from table 4, that the primer set BLSV-HEVf/BLSV-HEVr according to the invention is capable of giving a PCR-product in all cases, i.e. with all field isolates tested, regardless their geographical origin.

Finally it follows from this table that probe Probe HEV-3 (*vide infra*) is capable of detecting the PCR-product in all cases, i.e. with all field isolates tested, in a real-time PCR.

The method above thus provides a way of selectively detecting the presence or absence in a sample, of avian HEV, regardless the geographic origin of the HEV.

Thus, a first embodiment of the invention relates to a method for the detection of the presence of avian hepatitis E virus (avian HEV) in a sample, characterised in that said method comprises the steps of

5 a) performing a reverse transcriptase polymerase chain reaction (RT-PCR) of said sample using a primer set comprising a primer that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVf having a sequence as depicted in SEQ ID NO.: 5 and a primer that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVr having a

10 sequence as depicted in SEQ ID NO.: 6 and

b) examining the RT-PCR amplification result of step (a)

In a preferred form of this embodiment, one primer of the primer set is the oligonucleotide BLSV-HEVf having a sequence as depicted in SEQ ID NO.: 5 and the other primer of the primer set is the

15 oligonucleotide BLSV-HEVr having a sequence as depicted in SEQ ID NO.: 6.

One frequently used method for the detection of a specific nucleic acid sequence in a sample is known as a nested set PCR. Such a PCR reaction is comparable to the PCR reaction described above, but it uses the PCR product made by the first primer set as a template for a second PCR reaction.

In the nested set PCR reaction, one of the two primers of the second primer set can be a primer that comprises a stretch of at least 16 consecutive nucleotides from the oligonucleotide of Probe HEV-3 having a sequence as depicted in SEQ ID NO.: 8.

It was surprisingly found that this oligonucleotide also specifically binds to avian HEV cDNA, regardless the geographical origin of the avian HEV. This is even more remarkable because this oligonucleotide is slightly degenerate. Primers and probes on the basis of such degenerate oligonucleotides are in general slightly less selective due to their slightly degenerated character. In this case however, the degenerate character of this specific probe allows for selective binding only to avian HEV cDNA while at the same time allowing to bind to avian HEVs that slightly differ in the region to which this probe binds. Therefore,

25 this oligonucleotide in combination with the primer set according to the invention allows for a very specific detection of avian HEV regardless the geographical origin of that avian HEV.

In this nested set method, this oligonucleotide is used as a classical primer; so without the marker that is attached to the primer when it is used as a probe.

The PCR product that is made if the primers according to the invention detect avian HEV, stretches from position 4743 to 4918 (see table 2 for further explanation).

The oligonucleotide of the probe Probe HEV-3 anneals to the cDNA from position 4767-4788, which is close to the 5'-terminus of the cDNA.

Therefore, basically the only tool further needed for such a nested set PCR would be a second primer that is located close to the 3'-terminus of the cDNA, i.e. around position 4900. The use of a combination of the probe according to the invention as one primer and that second primer as the other primer would provide the basis for the nested set PCR.

5 Such a second primer can easily be designed by selecting an oligonucleotide e.g. around position 4900 and having a length of about 22 nucleotides, so e.g. from position 4897-4918, preferably an oligonucleotide having an AT/CG ratio is roughly comparable with that of the probe according to the invention. Again: Dieffenbach & Drexler; PCR primers, a laboratory manual. ISBN 0-87969-447-5 (1995) provide ample guidance when designing such a second primer.

10 The nested set PCR would result in a PCR product of which the length and amount can be analysed e.g. by means of conventional agarose or polyacrylamide gel electrophoresis. In case the PCR product obtained from the first PCR reaction is a false positive product, i.e. not of avian HEV origin, the second primer pair, formed e.g. by the oligonucleotide having a sequence as depicted SEQ ID No.: 8 and the second primer, would not, or at least not both, anneal to the first PCR product and thus there would be no PCR
15 product of the expected length be detected on gel.

The nested set method above provides a way of more selectively detecting the presence or absence in a sample, of avian HEV.

Therefore, a preferred form of this embodiment relates to a method for the detection of the presence of
20 avian hepatitis E virus (avian HEV) in a sample, characterised in that said method comprises the steps of
a) performing a reverse transcriptase polymerase chain reaction (RT-PCR) of said sample using a primer set comprising a primer that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVf having a sequence as depicted in SEQ ID NO.: 5 and a primer that
comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVr having a
25 sequence as depicted in SEQ ID NO.: 6, followed by the step of
b) performing a polymerase chain reaction (PCR) using a second primer set comprising a primer that
comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide having a sequence as
depicted in SEQ ID NO.: 8 and
c) examining the PCR amplification result of step b
30

In a more preferred form of this embodiment, one primer of the primer set is the oligonucleotide BLSV-HEVf having a sequence as depicted in SEQ ID NO.: 5 and the other primer of the primer set is the oligonucleotide BLSV-HEVr having a sequence as depicted in SEQ ID NO.: 6.

35 In another more preferred form of this embodiment, one primer of the second primer set is an oligonucleotide having the sequence as depicted in SEQ ID NO.: 8.

Basically, the function of step b) of the nested set PCR is to eliminate false positive signals, i.e.; in the unlikely case that the two primers of the first primer set according to the invention would pick up a non-

avian HEV sequence or any other sequence from an avian sample, such false positive signals should not give a reaction in step b). Moreover, in general a nested set PCR increases the sensitivity of the test.

5 An even more efficient and also selective method, that would avoid the additional efforts of designing a second primer, applying the nested set PCR and detecting the presence of the nested set PCR product on e.g. a gel, is the so-called real-time RT-PCR.

10 This method basically relies on the RT-PCR methods described above, but it does not even need the nested set PCR step. Nevertheless, it has a level of selectivity that compares to the nested set methods described herein. It relies only on the primer set according to the invention and a probe that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide Probe HEV-3 having a sequence as depicted in SEQ ID NO.: 8, according to the invention. In this method, the probe is an oligonucleotide with a fluorophore and a quencher molecule attached to it.

15 Several versions of such probes exist when it comes to the fluorophore or quencher used or the working mechanism behind the probe/quencher combination. Merely as an example, such probes are commercially available as TaqMan probes, Scorpions probes and Molecular Beacons probes (*vide infra*).

20 In this method using a probe, the detection can be done e.g. by using the probe according to the invention. This probe binds, as said above, in a selective manner with an internal sequence of the cDNA made using the primer set according to the invention. The probe anneals in step b) to the cDNA from position 4767-4788, which is close to the 5'-terminus of the cDNA. This however happens only in case the cDNA is indeed of avian HEV origin. Therefore, the probe according to the invention makes the detection of avian HEV more reliable than a mere RT-PCR reaction. In the unlikely case that the two selective primers would pick up a false positive signal, this would be noticed in step b/c, because the probe would not
25 anneal to it.

TaqMan based real-time RT-PCR methods are a development by Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404, USA.

TaqMan based real-time RT-PCR methods are described i.a. in literature references 8, 10 and 33.

30 Real-time RT-PCR methods based upon Scorpions and Molecular beacons are available through PREMIER Biosoft International, 3786 Corina Way, Palo Alto CA 94303-4504, USA.

The use of real-time PCR on the basis of Molecular Beacons is described in detail in i.a: Molecular Beacons; A New Tool to Identify Point Mutations and to Analyze Gene Expression in Mycobacterium tuberculosis by Manganelli, R., Tyagi, S. and Smith, I., in: Methods in Molecular Medicine, vol. 54: page
35 295-310, Mycobacterium Tuberculosis Protocols, Edited by: T. Parish and N. G. Stoker © Humana Press Inc., Totowa, NJ.

TaqMan probes such as the Probe HEV-3 consist of a fluorophore covalently attached to the 5'-end of the oligonucleotide probe and a quencher at the 3'-end. Several different fluorophores (e.g. 6-

carboxyfluorescein, acronym: FAM, or tetrachlorofluorescein, acronym: TET) and quenchers (e.g. tetramethylrhodamine, acronym: TAMRA, or dihydrocyclopyrroloindole tripeptide minor groove binder, acronym: MGB) are available. The quencher molecule quenches the fluorescence emitted by the fluorophore when excited by the cyclers' light source via FRET (Fluorescence Resonance Energy Transfer). As long as the fluorophore and the quencher are in proximity, quenching inhibits any fluorescence signals.

TaqMan probes are designed such that they anneal within a DNA region amplified by a specific set of primers. As Taq polymerase extends the primer and synthesizes the nascent strand, the 5' to 3' exonuclease activity of the polymerase degrades the probe that has annealed to the template. Degradation of the probe releases the fluorophore from it and breaks the close proximity to the quencher, thus relieving the quenching effect and allowing fluorescence of the fluorophore. Hence, fluorescence detected in the real-time PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR.

Taqman probes are preferred probes for use in the methods and diagnostic tools according to the invention.

In fact, the Taqman probe Probe HEV-3 described here is an oligonucleotide having a DNA sequence as depicted in SEQ ID NO.: 8, however with a fluorophore and a quencher attached to it. But as explained (*vide supra*), the probe can also be a shorter or longer oligonucleotide comprising a stretch of at least 16 consecutive nucleotides from an oligonucleotide Probe HEV-3 having a sequence as depicted in SEQ ID NO.: 8.

Since in this method both the annealing of the primers and the probe take place in one process, the development of a colour reaction takes place at practically the same moment as the DNA amplification. Therefore, such reaction is referred to as a real-time RT-PCR reaction.

Thus, another preferred form of this embodiment relates to a method for the detection of the presence of avian hepatitis E virus (avian HEV) in a sample, characterised in that said method comprises the steps of

a) performing a reverse transcriptase polymerase chain reaction (RT-PCR) of said sample using a primer set comprising a primer that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVf having a sequence as depicted in SEQ ID NO.: 5 and a primer that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVr having a sequence as depicted in SEQ ID NO.: 6 and

b) examining the RT-PCR amplification result of step (a) using a probe comprising a stretch of at least 16 consecutive nucleotides from an oligonucleotide Probe HEV-3 having a sequence as depicted in SEQ ID NO.: 8.

In a more preferred form of this embodiment, one primer of the primer set is the oligonucleotide BLSV-HEVf having a sequence as depicted in SEQ ID NO.: 5 and the other primer of the primer set is the oligonucleotide BLSV-HEVr having a sequence as depicted in SEQ ID NO.: 6.

5 In another more preferred form of this embodiment, the probe is Probe HEV-3.

This method according to the invention can be further improved by adding a so-called internal control RNA. In fact, the internal control is a parallel experiment, in which an amount of a control RNA is added to the test sample, as well as primers and a probe that are specific for that control RNA (IC-RNA). (Or
10 alternatively, though less preferred, the control RNA and the primers and probe are tested separately in a parallel RT-PCR test). Such primers and probe should be non-HEV related; if they would be HEV-related they might interfere with the HEV-specific part of the method.

It is clear that the colour of the fluorophore of the HEV-probe and the fluorophore of the non-HEV probe must be different, in order to discriminated between HEV-specific fluorescence and IC-RNA-specific
15 fluorescence.

Since all the components for a successful reaction showing the presence of the IC-RNA are present, there will be a specific fluorescence, indicating that the various process steps were successful. Preferably, the IC-RNA test is performed in the same test tube as the avian HEV-detection test. Thus, if fluorescence of the IC-RNA specific fluorophore is detected, the test as such is reliable, and if in addition fluorescence of
20 the HEV specific fluorophore is detected, that proves the presence of HEV-material.

If fluorescence of the IC-RNA specific fluorophore is detected, but no fluorescence of the HEV specific fluorophore is detected, that proves the absence of HEV-material.

If no fluorescence of the IC-RNA specific fluorophore is detected, the test is not reliable and should not be taken into account.

25 Thus, the use of an internal control is important to exclude false negative results due to e.g. incomplete RNA isolation, incomplete reverse transcriptase reaction or inhibition of PCR.

In principle, a synthetic RNA can be used as the starting material for the internal control. As an alternative to synthetic fragments of RNA, housekeeping genes or different genes of the host or from different
30 pathogens can be used as internal control. Their unknown and changing concentration, instability and bio safety concerns make them however more difficult to handle and integrate in the PCR assay than *in vitro* transcribed RNA (14).

For this reasons, a preferred internal control system is the universal heterologous internal control system designed by Hoffmann et al. (14). It is based on RNA and could easily be adapted and integrated in the
35 assay to check for successful RNA extraction and RT-PCR. As a longer product less inhibits amplification of target template (14), the primers for amplification of a 487 bp product were chosen ('AcGFP-13-F/AcGFP-10-R'). IC-RNA was added prior to RNA isolation, which resulted in a reliable amplification of internal control but did not inhibit amplification of avian HEV.

Thus, an even more preferred form of this embodiment relates to a method according to the invention, characterised in that the step of performing the real-time RT-PCR reaction of said method additionally uses at least one additional non-HEV related primer set and at least one additional non-HEV related probe.

5 If quantification of the PCR reaction is required, separate parallel tests can be run in which known amounts of HEV-RNA and the primers and probe according to the present invention are present. This would allow for standard curves to be drawn that provide a relation between the amount of RNA in the parallel test and the number of cycles required to reach the fluorescence detection threshold. These standard curves can then subsequently be used to determine the unknown amount of HEV-RNA in the
10 sample.

Therefore, a method according to the invention to which separate parallel tests are run in order to make standard curves that are subsequently used for the quantification of the amount of HEV-RNA in a sample, is referred to as a quantitative method.

15 Methods for the preparation of RNA for the internal control, as well as the preparation of standard amounts of HEV-RNA for making standard curves in parallel tests are given in the Example section under the heading "Generation of standard and internal control RNA".

It is clear that the biological material is preferably submitted to further purification steps.

20 Since the method for the detection of avian HEV is based upon viral RNA, this RNA is preferably purified from the sample to a certain extent. Purification in this respect means that material in the sample other than HEV-RNA is to a certain extent removed from the sample before the sample is submitted to a method according to the invention. Such purification can e.g. comprise de-proteinisation, removal of cell debris, removal of DNA, RNA-extraction and the like. An example of RNA purification from a sample is given in
25 literature reference 6. The Examples below also describe an efficient method to prepare purified RNA.

Thus, a still even more preferred form of the present invention relates to a method according to the invention, characterised in that said method comprises an RNA purification step preceding step a).

30 Another embodiment of the present invention relates to a primer set comprising a primer that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVf having a sequence as depicted in SEQ ID NO.: 5 and/or a primer that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVr having a sequence as depicted in SEQ ID NO.: 6

35 A further embodiment of the present invention relates to a primer set comprising a primer that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVf having a sequence as depicted in SEQ ID NO.: 5 and/or a primer that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVr having a sequence as depicted in SEQ ID NO.: 6 for use in the detection of the presence of avian HEV in a sample.

Still another embodiment of the present invention relates to a primer or a probe comprising a stretch of at least 16 consecutive nucleotides from an oligonucleotide Probe HEV-3 having a sequence as depicted in SEQ ID NO.: 8.

A still further embodiment of the present invention relates to a primer or a probe comprising a stretch of at least 16 consecutive nucleotides from an oligonucleotide Probe HEV-3 having a sequence as depicted in SEQ ID NO.: 8 for use in the detection of the presence of avian HEV in a sample.

Again another embodiment of the present invention relates to diagnostic test kits for the detection of the presence of avian hepatitis E virus (avian HEV) in a sample. Such kits allow for the methods according to the invention to be practised.

Thus, a first form of this second embodiment relates to a diagnostic test kit for the detection of the presence of avian hepatitis E virus (avian HEV) in a sample, characterised in that said kit comprises at least a primer set comprising a primer comprising a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVf having a sequence as depicted in SEQ ID NO.: 5 and a primer comprising a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVr having a sequence as depicted in SEQ ID NO.: 6

Another form of this second embodiment relates to diagnostic test kit for the detection of the presence of avian hepatitis E virus (avian HEV) in a sample, characterised in that said kit comprises at least a primer set comprising a primer comprising a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVf having a sequence as depicted in SEQ ID NO.: 5 and a primer comprising a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVr having a sequence as depicted in SEQ ID NO.: 6 and at least a primer or a probe comprising a stretch of at least 16 consecutive nucleotides from an oligonucleotide Probe HEV-3 having a sequence as depicted in SEQ ID NO.: 8.

These various primers and probes may be present in the kit in separate vials. They may also be present in one and the same vial. They could, for ease of manipulation and in order to avoid unnecessary risk of contamination, even be present in the test vial to which the sample is added (with the exception of the HEV-RNA and primers and probe that are used for making the standard curves for quantification, see below; these experiments are separately run in parallel).

They would preferably be present in a dried form, in order to keep them stable under room storage conditions.

A diagnostic test kit according to the invention may additionally comprise a reverse transcriptase and/or a thermo stable DNA polymerase. These enzymes are necessary to perform a real-time RT-PCR and for ease of use they might thus already be incorporated in the diagnostic test kit.

If an internal control of the real-time RT-PCR is required, a second set of primers and probe, in this case non-HEV primers and a non-HEV probe as discussed above, as well as the IC-RNA may be included in the diagnostic test kit. It goes without saying that the four DNA building blocks and the necessary buffers may additionally be included in the diagnostic test kit as well.

If quantification of the PCR reaction is required, parallel tests can be run in which known amounts of HEV-RNA and the primers and probe according to the present invention are present. This would allow for standard curves to be drawn that provide a relation between the amount of RNA in the parallel test and the number of cycles required to reach the fluorescence threshold. These standard curves can then be used to determine the amount of HEV-RNA in the sample. Therefore, preferably the diagnostic test kit additionally comprises known amounts of HEV-RNA that allow for quantification to be made.

Hereunder, examples of how to perform the method according to the invention are given. It goes without saying that the examples should not be considered to limit the scope of the invention in any way.

1 EXAMPLES

Example 1

1 Experiments

1.1 Samples

A set of different samples was used to develop the new duplex real-time RT-PCR assay and compared to conventional RT-PCR (Table 1). The selected samples were mostly obtained from birds showing clinical signs for HSS or BLSD and represent different geographical origins and genotypes (21).

1.2 RNA isolation and addition of internal control

RNA isolation was done accordingly to the 'Solution D' method (6), except that 96 % ethanol was used instead of 2-propanol for both precipitation steps (-80°C for 1 h, -20°C over night). If RNA isolation was performed with the use of internal control RNA, samples were first mixed with Solution D (lysis buffer) and then the internal control RNA was added. Further steps followed the usual protocol of the 'Solution D' method. Pellets were resuspended in 40 µl ultra purified water and RNA was stored at -80°C.

1.3 Primers and probes

Primers and probes used in this study are listed in table 2. Primer pairs 'Helicase F/Helicase R' (15) and 'Forw1_C-BLSV/Rev1_C-BLSV' (2) were used for conventional RT-PCR. Primer pair 'BLSV-

HEVf/BLSV-HEVr' and probe 'Probe ORF3-HEV' were designed in this study and used for real-time RT-PCR. In order to design these primers and probe four almost complete genome sequences of avian HEV (GenBank accession numbers AM943646, AM943647, AY535004, EF206691) were aligned using software Accelrys Gene, version 2.5 (Accelrys, San Diego, CA, USA). These primers and probes were designed to anneal within the relatively conserved ORF3 region of avian HEV. In addition, 'BLSV-HEVf/BLSV-HEVr' PCR products of nine field samples were sequenced (GenBank accession numbers FN995640 to FN995648) and a degenerate probe, named 'Probe HEV-3', was designed (Table 2).

Primers and probe of the universal IC system designed by Hoffmann et al. (14) were slightly adapted to the sequence of the vector AcGFP1-C1 (Clontech Laboratories, Inc., Mountain View, CA, USA) (Table 2).

Specificity of primers and probes was tested in a BLAST search. All primers and probes were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

1.4 Conventional RT-PCR

The conventional RT-PCR for amplification of avian HEV was performed as described before (21). For generating a PCR product using vector AcGFP1-C1 (Clontech Laboratories, Inc., Mountain View, CA, USA), illustraTM PuReTaqTM Ready-To-GoTM PCR beads (GE Healthcare, Little Chalfont, BUCKS, UK) were used with a primer concentration of 1 µM for each primer 'AcGFP-15-F' and 'AcGFP-10-R'. The temperature-time profile was: 95°C for 5 min, 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and finally 72°C for 7 min. The PCR products were run on a 2 % agarose gel at 100 volts for 50 minutes and visualized on a UV illuminator.

1.5 Generation of standard and internal control RNA (HEV-RNA and IC-RNA)

For optimization of real-time RT-PCR, generation of standard curves and internal control, *in vitro* transcribed RNA was used. From each of the two field isolates, one from Hungary (05/5492) and one from Australia (06/561), a 176 bp RT-PCR product (primer pair 'BLSV-HEVf/BLSV-HEVr', Table 2), was cloned into pCR4-TOPO vector using a TOPO TA Cloning[®] Kit for Sequencing (Invitrogen, Carlsbad, CA, USA). For generation of the internal control RNA a 712 bp PCR product (primer pair 'AcGFP-15-F/AcGFP-10-R', Table 2) of the GFP gene using standard vector AcGFP1-C1 (Clontech Laboratories, Inc., Mountain View, CA, USA) was amplified and cloned. Positive clones were sequenced at Eurofins MWG Operon and clones of correct sequence and orientation were chosen for further processing.

The vectors were linearized using *Pst*I restriction enzyme (Invitrogen, Carlsbad, CA, USA) and run on a 1 % agarose gel for confirmation and purification. Bands of correct size were excised and cleaned using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. The purified plasmid DNA was then *in vitro* transcribed using the MAXIscript[®] T7 kit (Ambion, Inc., Austin, TX, USA) according to manufacturer's protocol, except for adding 1 µl RNaseOUT (Invitrogen, Carlsbad, CA, USA) to the transcription reaction, which was performed for 1 hour at 37°C. The *in vitro* transcribed RNA was treated with TURBO DNA-freeTM Kit (Ambion, Inc., Austin, TX, USA) to completely remove residual DNA and precipitated with ammonium acetate and ethanol. The concentration of RNA was

measured at least four times by a spectrophotometer (SmartSpec Plus, Bio-Rad Laboratories, Inc., Hercules, CA, USA) and copies per μl were calculated using the mean values and the formula:
$$\text{copies}/\mu\text{l} = [(\text{g}/\mu\text{l RNA})/(\text{length (nt)} \times 340)] \times 6.022 \times 10^{23}.$$

5 1.6 Real-time RT-PCR

Real-time RT-PCR was performed in 25 μl on Rotor-Gene Q (Qiagen, Hilden, Germany) using TaqMan detection method with QuantiTect[®]Virus +ROX Vial Kit (Qiagen, Hilden, Germany). To include the internal control in the assay, additional to the primers and probe for avian HEV, primers and probe for amplification and detection of IC-RNA were included in the mastermix. The final mix was named 'HEV-3/IC' and consisted of primers 'BLSV-HEVf', 'BLSV-HEVr', 'AcGFP-13-F' and 'AcGFP-10-R', each in a final concentration of 0.4 μM . The probes 'Probe HEV-3' and 'Probe AcGFP' were added to the primer-probe mix 'HEV-3/IC' in a final concentration of 0.2 μM for each probe.

Duplex real-time RT-PCR was performed measuring simultaneously yellow and green fluorescence emitted by the two probes. The conditions for RT-PCR were as follows: 50°C for 30 min, 95°C for 5 min, 15 50 times of three-step cycling with 95°C for 15 sec, 60°C for 75 sec and 72°C for 15 sec. Data analysis was done by Rotor-Gene Q software version 1.7 (Qiagen, Hilden, Germany) by setting the threshold automatically. In order to quantify samples of unknown concentration, their Ct values were compared with the standard curve and the copies of avian HEV RNA per reaction were calculated. Real-time RT-PCR products were run on a 2 % agarose gel to test if products of correct size were amplified. Reverse transcriptase control reactions were included in order to confirm that the *in vitro* transcribed RNAs were not contaminated with residual DNA.

1.7 Standard curves and internal control

For generating standard curves ten-fold dilution series of *in vitro* transcribed RNA from 'BLSV-HEVf/BLSV-HEVr' PCR product (HEV-RNA) were prepared, starting from a concentration of 2.89×10^{12} copies of HEV-RNA per reaction. All standard curves consisted of at least five different concentrations, each of them run in duplicate.

For amplification of IC-RNA the primer pair 'AcGFP-13-F/AcGFP-10-R', that amplifies a 487 bp PCR product, was tested with dilution series of *in vitro* transcribed IC-RNA. To determine the amount of IC-RNA to be added to standard curve, dilution series of IC-RNA had been made and the least amount detected together with HEV-RNA reliably was chosen for use in the future assay. Ultimately, the ten-fold dilution series of *in vitro* transcribed HEV-RNA were spiked with 5.7×10^5 copies of *in vitro* transcribed IC-RNA. Since in the course of RNA isolation a certain amount of RNA is lost, 5.7×10^7 copies of IC-RNA were added to the samples to check the efficiency of RNA isolation.

35

1.8 Real-time RT-PCR sensitivity

The lowest dilution detected in standard curve was further diluted two-fold to determine the limit of detection of *in vitro* transcribed HEV-RNA. Additionally, a sample RNA (07/861) was serially diluted ten-fold, TaqMan real-time RT-PCR (primer-probe mix 'HEV-3/IC') and conventional RT-PCR (primer

pairs 'Helicase F/Helicase R' and 'Forw1_C-BLSV/Rev1_C-BLSV') were performed and the results were compared.

1.9 Real-time RT-PCR specificity

5 To test the specificity of the TaqMan real-time RT-PCR assay, it was performed with HEV positive samples of swine, wild boar (genotype 3, isolate wbGER27, GenBank accession number FJ705359) (29), rat (isolate R63, GenBank accession number GU345042) (18) and different avian pathogens. These were avian leukosis virus (RNA and DNA isolated from tissue culture), Marek's disease virus (DNA isolated from a positive field sample), avian reovirus (RNA isolated from vaccine) and fowl adenovirus (DNA
10 isolated from tissue culture). A sample of RNA isolated from the liver of a non-infected SPF chicken was also tested. Internal control RNA was added to all the samples.

2 Results

2.1 Optimization of real-time RT-PCR assay

15 Different primer and probe concentrations and temperature-time-profiles were tested. Adjusting primer and probe concentrations did not have much influence. Therefore the concentrations were chosen according to manufacturer's instruction for the QuantiTect® Virus +ROX Vial Kit (Qiagen, Hilden, Germany), which were 0.4 µM for the primers and 0.2 µM for the probes. The temperature-time profile was adapted, especially a longer reverse transcription step (30 min instead of 20 min) and a three-step
20 cycling protocol instead of two-step cycling protocol were crucial with regard to sensitivity of the new assay (data not shown).

First, nine field samples were tested in real-time RT-PCR with primer pair 'BLSV-HEVf/BLSV-HEVr' and probe 'Probe ORF3-HEV'. It appeared that from one sample (07/9643-2) no fluorescence was detected (section 2.5 *vide infra*) although a band was visible in gel electrophoresis performed to control
25 real-time RT-PCR (data not shown). 'BLSV-HEVf/BLSV-HEVr' PCR products of all nine samples were sequenced (Supplementary data), and the degenerate probe 'Probe HEV-3' was designed.

2.2 Standard curves and internal control

30 The duplex real-time RT-PCR assay showed a range over ten orders of magnitude (2.89×10^{12} to 2.89×10^3 copies per reaction). It has to be mentioned that the smallest dilution (2.89×10^3 copies per reaction) was not detected reliably and mostly did not fit well into the standard curve. If this dilution was not taken into account, an efficiency of 1.04 and a regression square of 0.996 was achieved. The IC-RNA added to standard curve was detected reliably with an average Ct of 30.17 and a standard deviation of 0.79 (data not shown).

35 By comparing a standard curve obtained from a dilution series of HEV-RNA without IC-RNA to a standard curve obtained from a dilution series of HEV-RNA together with IC-RNA, it could be shown that amplification of HEV-RNA was not inhibited by presence of IC-RNA. The standard curve without IC-RNA had an efficiency of 0.90 and a regression coefficient of 0.9964. If IC-RNA was included in the

same dilution series of HEV-RNA an efficiency of 0.94 and a regression square of 0.9958 was achieved for the standard curve.

Eight field samples were quantified by intercalating their Ct value to standard curve to demonstrate that the real-time RT-PCR for detection of avian HEV is suited for quantification of viral RNA present in these samples. Ct values between 22.48 and 28.97 were measured, corresponding to 2×10^7 to 2.92×10^5 copies of avian HEV RNA per sample (Table 3).

2.3 Assay sensitivity

The concentration of 2.89×10^4 copies of *in vitro* transcribed HEV-RNA per reaction was further diluted two-fold and duplex real-time RT-PCR reactions were run in duplicate. All concentrations were tested positively, but from the smallest dilution (1.8×10^3 copies per reaction) only in one of two reactions a Ct value was obtained. Therefore, a minimum of 3.6×10^3 copies can be detected reliably with this duplex real-time RT-PCR assay (data not shown).

RNA of sample 07/861A was diluted five times ten-fold and conventional and real-time RT-PCRs were performed. In conventional RT-PCR the dilutions up to 10^{-3} were detected using both primer pairs, 'Helicase F/Helicase R' and 'Forw1_C-BLSV/Rev1_C-BLSV'. When performing real-time RT-PCR the same sensitivity was determined. The undiluted sample gave almost the same Ct as the dilution 10^{-1} (30.47 and 30.92, respectively), probably due to presence of PCR inhibitors in the undiluted sample. For the dilution 10^{-2} a Ct of 33.91 was measured. The Ct of the last dilution detected (10^{-3} with a Ct of 40.21) was high, indicating that the assay reached the limit of detection. For the dilutions 10^{-4} and 10^{-5} amplification did not reach threshold level and no Ct was measured.

2.4 Assay specificity

The duplex real-time RT-PCR was performed with samples positive for swine HEV, wild boar HEV, rat HEV, avian leucosis virus, Marek's disease virus, avian reovirus, fowl adenovirus and a sample of RNA isolated from the liver of an SPF chicken. All samples were negative, i.e. no Ct was measured and no band was visible after gel electrophoresis. The internal controls and the avian HEV positive control reaction were positive.

2.5 Sample detection

The real-time RT-PCR assay based on the degenerate probe could detect all of the 16 samples tested (Table 4). In conventional RT-PCR nine out of 16 samples were detected with both primer pairs ('Helicase F/Helicase R' and 'Forw1_C-BLSV/Rev1_C-BLSV'). Four additional samples were detected with primer pair 'Helicase F/Helicase R', but not with 'Forw1_C-BLSV/Rev1_C-BLSV'. Three samples were detected by neither of the two primer pairs in conventional RT-PCR (Table 4).

3 Conclusion

When compared to conventional PCR, probe-based detection systems such as TaqMan have an improved specificity (13). The degenerate probe 'Probe HEV-3' could detect all samples tested in this

study. Among these were samples that were negative in conventional RT-PCR. Therefore, it was concluded that the real-time RT-PCR based on 'BLSV-HEVf/BLSV-HEVr' primers and 'Probe HEV-3' is highly suited for detecting avian HEV samples of different geographical origins and genotypes. The samples were obtained from poultry in different European countries and Australia and were mainly classified within genotypes 1 and 3 of avian HEV (21). One sample belonging to genotype 2, according to analysis of helicase gene sequence, was also successfully tested with the real-time RT-PCR method. Therefore, the real-time RT-PCR according to the invention should also be suited for samples representing all different genotypes reported so far.

In vitro transcribed RNA was used as standard for quantification of avian HEV RNA, which is important because the reverse transcription step is limiting and amplification of RNA is not proportional to amplification of DNA, e.g. amplification of a plasmid used as standard. The method according to the invention detected approximately 3.6×10^3 copies of *in vitro* transcribed HEV RNA. The real-time RT-PCR for detection of avian HEV had a sensitivity similar to conventional RT-PCR using ten-fold dilutions of sample RNA. Not all samples were detected in conventional RT-PCR, although two primer pairs, binding within ORF1 and ORF2, were used. The real-time RT-PCR presented here, has the advantage to detect a large range of samples from different genotypes in a single procedure step with only one primer pair, having the same sensitivity as conventional RT-PCR. Furthermore, it was possible to quantify the samples tested in this study by use of a standard curve consisting of *in vitro* transcribed RNA. All Ct values of the samples were in range of the standard curve and the amount of avian HEV present in each sample could be expressed as copies of avian HEV RNA per reaction. If quantification of samples is done, it must be considered that the values are theoretical and need normalization based on equal quantity and quality of starting material (9). The quantification of different samples in this study was done primarily to demonstrate that the real-time RT-PCR for quantification of avian HEV could be used for quantification of various types of sample material, e.g. in studies about pathogenicity of avian HEV.

It is concluded that the TaqMan real-time RT-PCR method according to the invention is suitable for universal detection of avian HEVs. The same was found to be true for the TaqMan duplex real-time RT-PCR method according to the invention. Beside the detection of different genotypes of avian HEV with only one primer pair in heterologous sample material, it provides essential features of real-time RT-PCR. Among these features are quantification, inclusion of an internal control when desired and a reduced contamination risk due to closed-tube procedures and one-step reaction. The real-time RT-PCR method according to the invention is a specific and sensitive diagnostic tool for universal detection of avian HEV in animal samples.

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36. **Dieffenbach & Drexler**; PCR primers, a laboratory manual. ISBN 0-87969-447-5 (1995).

Claims.

- 1) A method for the detection of the presence of avian hepatitis E virus (avian HEV) in a sample, characterised in that said method comprises the steps of
 - a) performing a reverse transcriptase polymerase chain reaction (RT-PCR) of said sample using a primer set comprising a primer that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVf having a sequence as depicted in SEQ ID NO.: 5 and a primer that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVr having a sequence as depicted in SEQ ID NO.: 6 and
 - b) examining the RT-PCR amplification result of step (a)
- 2) A method for the detection of the presence of avian HEV according to claim 1, characterised in that said method comprises the steps of
 - a) performing a reverse transcriptase polymerase chain reaction (RT-PCR) of said sample using a primer set comprising a primer that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVf having a sequence as depicted in SEQ ID NO.: 5 and a primer that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVr having a sequence as depicted in SEQ ID NO.: 6, followed by the step of
 - b) performing a polymerase chain reaction (PCR) using a second primer set comprising a primer that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide Probe HEV-3 having a sequence as depicted in SEQ ID NO.: 8 and
 - c) examining the PCR amplification result of step b
- 3) A method for the detection of the presence of avian HEV according to claim 1, characterised in that said method comprises the steps of
 - a) performing a reverse transcriptase polymerase chain reaction (RT-PCR) of said sample using a primer set comprising a primer that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVf having a sequence as depicted in SEQ ID NO.: 5 and a primer that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVr having a sequence as depicted in SEQ ID NO.: 6 and
 - b) examining the RT-PCR amplification result of step (a) using a probe comprising a stretch of at least 16 consecutive nucleotides from an oligonucleotide Probe HEV-3 having a sequence as depicted in SEQ ID NO.: 8.
- 4) A method according to any of claims 1-3, characterised in that the step of performing the RT-PCR reaction of said method additionally uses at least one additional non-HEV related primer set and at least one additional non-HEV related probe.

- 5) A method according to any of claims 1-4, characterised in that said method comprises an RNA purification step preceding step a).
- 5 6) A primer set comprising a primer that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVf having a sequence as depicted in SEQ ID NO.: 5 and/or a primer that comprises a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVr having a sequence as depicted in SEQ ID NO.: 6
- 10 7) A primer or a probe comprising a stretch of at least 16 consecutive nucleotides from an oligonucleotide Probe HEV-3 having a sequence as depicted in SEQ ID NO.: 8.
- 15 8) Diagnostic test kit for the detection of the presence of avian hepatitis E virus (avian HEV) in a sample, characterised in that said kit comprises at least a primer set comprising a primer comprising a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVf having a sequence as depicted in SEQ ID NO.: 5 and a primer comprising a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVr having a sequence as depicted in SEQ ID NO.: 6
- 20 9) Diagnostic test kit for the detection of the presence of avian HEV according to claim 8, characterised in that said kit comprises a) at least a primer set comprising a primer comprising a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVf having a sequence as depicted in SEQ ID NO.: 5 and a primer comprising a stretch of at least 16 consecutive nucleotides from an oligonucleotide BLSV-HEVr having a sequence as depicted in SEQ ID NO.: 6 and b) at least a primer or a probe comprising a stretch of at least 16 consecutive
- 25 nucleotides from an oligonucleotide Probe HEV-3 having a sequence as depicted in SEQ ID NO.: 8.
- 30 10) Diagnostic test kit for the detection of the presence of avian HEV according to claim 8 or 9, characterised in that said test kit comprises at least one additional non-HEV related primer set and at least one additional non-HEV related probe.

TABLE 1. Samples used in this study.

Sample number	Geographical origin	Genotype	Material
05/2294	Australia	n.a.	Antigen from liver homogenate
05/5492	Europe (Hungary)	3 ^a	Cloacal swab
05/5493	Europe (Hungary)	n.a.	Cloacal swab
05/5495	Europe (Hungary)	n.a.	Bile pooled from 3 birds
05/6738	Australia	n.a.	Pooled liver homogenate
05/6744-1 ^c	Australia (New South Wales)	1 ^a	Crude virus spotted on FTA card
05/6744-2 ^c	Australia (New South Wales)	1 ^a	Crude virus spotted on FTA card
05/6744-3 ^c	Australia (New South Wales)	1 ^a	Crude virus spotted on FTA card
05/6744-4 ^c	Australia (Western Australia)	1 ^a	Crude virus spotted on FTA card
05/6745-2 ^c	Australia	1 ^a	Crude virus spotted on FTA card
06/561	Australia (New South Wales)	1 ^a	Liver smears spotted on FTA card
06/4582-1	Europe (Germany)	3 ^a	Liver
07/861 ^c	Europe (Poland)	3 ^a	Liver smears spotted on FTA card
07/9643-2 ^c	Europe (Poland)	3 ^a	Liver smears spotted on FTA card
08/9107	n.a.	n.a.	Liver homogenate
08/18764 ^c	n.a.	n.a.	Liver smears spotted on FTA card
10/243 ^c	n.a.	2 ^b	Liver smears spotted on FTA card

^a according to Marek et al. (2010)^b based on analysis of sequence data of 'Helicase F/Helicase R' RT-PCR product and phylogenetic tree (data not shown)5 ^c HEV region sequenced in this study, see Supplementary data

n.a. – not available

TABLE 2. Primers and probes for avian HEV and internal control used in this study. Nucleotides that differ to the
10 reference primer and probe sequences are written in bold letters.

Primer/Probe	Sequence 5'-3'	Position ^a	Reference
Helicase F	TGG CGC ACY GTW TCY CAC CG	2795-2814	Huang et al. (2002)
Helicase R	CCT CRT GGA CCG TWA TCG ACC C	2981-2960	Huang et al. (2002)
Forw1_C-BLSV	GGT ATG GTT GAT TTT GCC ATA AAG	5437-5460	Bilic et al. (2009)
Rev1_C-BLSV	GCT GCN CGN ARC AGT GTC GA	5717-5698	Bilic et al. (2009)
BLSV-HEVf	AAT GTG CTG CGG GGT GTC AA	4743-4762	this study
BLSV-HEVr	CAT CTG GTA CCG TGC GAG TA	4918-4899	this study
Probe ORF3-HEV	FAM-CTC CCA AAC GCT CCC AGC CGG A-BHQ1	4767-4788	this study
Probe HEV-3	FAM-CTC CCA AAC GCY CYC AGC CGG A-BHQ1	4767-4788	this study
AcGFP-15-F	GAG CAA GGG CGC CGA GC	618-634	Hoffmann et al. (2006)
AcGFP-13-F	TCA CAT GAA GCA GCA CGA C	843-861	Hoffmann et al. (2006)
AcGFP-10-R	CTT GTA CAG CTC ATC CAT GC	1329-1310	Hoffmann et al. (2006)
Probe AcGFP	HEX-TCC ACC CAG AGC GCC CTG TCC A-BHQ1	1219-1240	Hoffmann et al. (2006)

^a according to EaHEV (GenBank accession number AM943646) and sequence file of pAcGFP1-C1 (Clontech Laboratories, Inc.), respectively

TABLE 3. Samples quantified by real-time RT-PCR using primer-probe mix 'HEV-3/IC'. All threshold cycle (Ct) values measured were in range of standard curve and the amount of copies of avian HEV RNA per reaction was determined.

Sample number	Ct	Copies of avian HEV RNA/reaction
05/2294	28.97	2.92E+05
05/5492	28.58	4.06E+05
05/6738	26.89	1.21E+06
05/6744-2	23.68	9.65E+06
05/6744-3	22.48	2.10E+07
05/6744-4	25.61	2.77E+06
05/6745-2	28.10	5.51E+05
08/9107	25.79	2.27E+06

5

TABLE 4. Conventional vs. real-time RT-PCR using a set of field samples as templates. Conventional RT-PCRs were performed with two different primers pairs: 'Helicase F/Helicase R' and 'Forw1_C-BLSV/Rev1_C-BLSV'. Real-time RT-PCRs were performed with primer pair 'BLSV-HEVf/BLSV-HEVr' and two different probes: 'Probe ORF3-HEV' and 'Probe HEV-3'.

Sample number	Conventional RT-PCR		Real-time RT-PCR	
	Primers 'Helicase F/Helicase R'	Primers 'Forw1_C-BLSV/Rev1_C-BLSV'	Primers 'BLSV-HEVf/BLSV-HEVr'	
			Probe 'ORF3-HEV'	Probe 'HEV-3'
05/2294	+	+	n.a.	+
05/5492-4	+	-	n.a.	+
05/5493-4	-	-	n.a.	+
05/5495-3	+	+	n.a.	+
05/6738	+	+	n.a.	+
05/6744-1	+	+	+	+
05/6744-2	+	+	+	+
05/6744-3	+	+	+	+
05/6744-4	+	+	+	+
05/6745-2	+	+	+	+
06/4582-1	-	-	n.a.	+
07/861	+	+	+	+
07/9643-2	-	-	-	+
08/18764	+	-	+	+
10/243	+	-	+	+
08/9107	+	-	n.a.	+
no. of samples tested positive/ no. of samples tested	13/16	9/16	8/9	16/16

10

n.a. – not available; + = positive result; - = negative result

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/051723

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/70
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, Sequence Search, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/053712 A2 (VIRGINIA TECH INTELL PROP [US]) 11 July 2002 (2002-07-11)	1,5-10
Y	page 7, line 30 - page 8, line 7; claims 19,20 figures 9A-9C, 17 page 26, line 24 - line 30 page 28, line 6 - line 27 -& DATABASE EMBL [Online] 27 November 2002 (2002-11-27), "Sequence 1 from Patent W002053712.", XP002639738, retrieved from EBI accession no. EMBL:AX556468 Database accession no. AX556468 -/--	2-4



Further documents are listed in the continuation of Box C.



See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

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23/03/2012

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/051723

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	-& DATABASE EMBL [Online] 27 November 2002 (2002-11-27), "Sequence 9 from Patent W002053712.", XP002639739, retrieved from EBI accession no. EMBL:AX556476 Database accession no. AX556476 -----	
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/051723

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HOFFMANN B ET AL: "A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses", JOURNAL OF VIROLOGICAL METHODS, ELSEVIER BV, NL, vol. 136, no. 1-2, 1 September 2006 (2006-09-01), pages 200-209, XP025030292, ISSN: 0166-0934, DOI: DOI:10.1016/J.JVIROMET.2006.05.020 [retrieved on 2006-09-01] cited in the application abstract	3,4
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A	----- JOHNE REIMAR ET AL: "Detection of a novel hepatitis E-like virus in faeces of wild rats using a nested broad-spectrum RT-PCR", JOURNAL OF GENERAL VIROLOGY, vol. 91, no. Part 3, March 2010 (2010-03), pages 750-758, XP002639743, ISSN: 0022-1317 cited in the application abstract	1-5
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/051723

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X,P	TROXLER S ET AL: "Detection and quantification of avian hepatitis E virus from clinical samples by a new Taqman real-time PCR including an internal control", INTERNATIONAL MEETING ON EMERGING DISEASES AND SURVEILLANCE 2011,, 4 February 2011 (2011-02-04), pages 153-154, XP009148939, the whole document -----	1-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2012/051723

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 02053712	A2	11-07-2002	
		US 2003059873 A1	27-03-2003
		US 2005255461 A1	17-11-2005
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