TaqMan Real-Time Reverse Transcription-PCR Assay for Universal Detection and Quantification of Avian Hepatitis E Virus from Clinical Samples in the Presence of a Heterologous Internal Control RNA^{∇†}

Salome Troxler, Ana Marek, Irina Prokofieva, Ivana Bilic, and Michael Hess*

Clinic for Avian, Reptile and Fish Medicine, Department for Farm Animals and Veterinary Public Health,
University of Veterinary Medicine, Vienna, Austria

Received 12 August 2010/Returned for modification 1 November 2010/Accepted 11 January 2011

Avian hepatitis E virus (HEV) isolates could be separated into at least three genotypes. In this study, the development of the first duplex TaqMan real-time reverse transcription-PCR (RT-PCR) assay for detection and quantification of avian HEV is presented. Primers and probes binding within relatively conserved open reading frame 3 (ORF3) were designed. Tenfold dilution series of *in vitro*-transcribed avian HEV RNA were used as the standard for quantification. A 712-bp region of the green fluorescent protein gene was transcribed *in vitro* and used as a heterologous internal control for both RNA isolation and real-time RT-PCR. The duplex real-time RT-PCR for avian HEV had an efficiency of 1.04, a regression squared value of 0.996, and a sensitivity of approximately 3.6×10^3 copies per reaction mixture when *in vitro*-transcribed RNA was used as the template. The presence of *in vitro*-transcribed heterologous internal control RNA did not affect amplification of avian HEV RNA compared to that achieved by the single assay. The sensitivity of the real-time RT-PCR assay was comparable to that of conventional RT-PCR, and it was shown to be highly specific, as tissues from uninfected chickens, mammalian HEVs, and other viral genomes did not produce positive signals. All tested field samples with virus belonging to different avian HEV genotypes were successfully detected with this new duplex TaqMan real-time RT-PCR assay.

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

Avian HEV has so far not been isolated from species other than the chicken. It was experimentally 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 geographical distribution pattern was observed (2, 21). Recently, HEVs detected in Norway rats were genetically identified (18).

Due to problems in efficiently propagating hepatitis E virus in cell culture, the diagnosis is based on antibody tests (enzyme-linked immunosorbent assay [ELISA], agar gel immuno-

diffusion) and reverse transcription-PCR (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 by using a truncated capsid protein of avian HEV, and its cross-reactivity with human and swine HEVs was shown (11). On the basis of this ELISA, several studies about the seroprevalence of avian HEV were carried out (15, 28, 30). A few RT-PCRs for avian HEV are described (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.

The genomic organization of avian HEV is similar to that of human and swine HEV, both consisting of three open reading frames (ORFs) and two short noncoding regions (NCRs). The length of the avian HEV genome (6.6 kb) is about 600 bp shorter than that of the 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, and a real-time RT-PCR method for detection of avian HEV is needed.

Real-time RT-PCR has become an essential diagnostic technique in virology that provides advantages such as shorter detection times, improved sensitivity and specificity, closed-tube procedures, high throughputs, and the possibility to quantify the viral material present in the sample. Detection of the target via binding of a probe such as TaqMan is more specific than that via binding of SYBR green. Especially for RNA viruses that have highly variable genomes, the choice of appropriate primer and probe binding sites is crucial. An addi-

* Corresponding author. Mailing address: Clinic for Avian, Reptile and Fish Medicine, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Vienna, Austria. Phone: 43 1250775150. Fax: 43 1250775192. E-mail: michael.hess@vetmeduni.ac.at.

† Supplemental material for this article may be found at <http://jcm.asm.org/>.

[∇] Published ahead of print on 9 February 2011.

TABLE 1. Samples used in this study

Sample no.	Geographical origin	Genotype	Material
05/2294	Australia	NA ^a	Antigen from liver homogenate
05/5492	Europe (Hungary)	3 ^b	Cloacal swab
05/5493	Europe (Hungary)	NA	Cloacal swab
05/5495	Europe (Hungary)	NA	Bile pooled from 3 birds
05/6738	Australia	NA	Pooled liver homogenate
05/6744-1 ^d	Australia (New South Wales)	1 ^b	Crude virus spotted on FTA card
05/6744-2 ^d	Australia (New South Wales)	1 ^b	Crude virus spotted on FTA card
05/6744-3 ^d	Australia (New South Wales)	1 ^b	Crude virus spotted on FTA card
05/6744-4 ^d	Australia (Western Australia)	1 ^b	Crude virus spotted on FTA card
05/6745-2 ^d	Australia	1 ^b	Crude virus spotted on FTA card
06/561	Australia (New South Wales)	1 ^b	Liver smears spotted on FTA card
06/4582-1	Europe (Germany)	3 ^b	Liver
07/861 ^d	Europe (Poland)	3 ^b	Liver smears spotted on FTA card
07/9643-2 ^d	Europe (Poland)	3 ^b	Liver smears spotted on FTA card
08/9107	NA	NA	Liver homogenate
08/18764 ^d	NA	NA	Liver smears spotted on FTA card
10/243 ^d	NA	2 ^c	Liver smears spotted on FTA card

^a NA, not available.^b According to Marek et al. (21).^c On the basis of analysis of sequence data of the Helicase F/Helicase R RT-PCR product and phylogenetic tree (data not shown).^d HEV region sequenced in this study (see data in the supplemental material).

tional advantage of TaqMan real-time PCR is the possibility to perform multiplex PCR and to include an internal control (IC) in the samples to avoid false-negative results due to incomplete RNA extraction or the presence of common PCR inhibitors described for clinical samples (13).

The aim of this study was to develop a TaqMan duplex real-time RT-PCR for universal and quantitative detection of avian HEV including a heterologous internal control system based on *in vitro*-transcribed RNA. The sensitivity of the new system was compared to that of conventional RT-PCR, and its specificity was tested on samples of mammalian HEVs, other viral chicken pathogens, and uninfected tissues. The developed method was tested on clinical samples of avian HEV of different geographical origins belonging to different genotypes.

MATERIALS AND METHODS

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

RNA isolation and addition of an 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 overnight). 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 ultrapurified water, and RNA was stored at −80°C.

Primers and probes. The 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 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, and EF206691) were aligned using the software Accelrys Gene, version 2.5 (Accelrys, San Diego, CA). 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 and a degenerate probe, named HEV-3, was designed (Table 2).

The primers and probes 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) (Table 2).

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

TABLE 2. Primers and probes for avian HEV and internal control used in this study

Primer or probe	Sequence (5'–3') ^a	Position ^b	Reference
Helicase F	TGG CGC ACY GTW TCY CAC CG	2795–2814	Huang et al. (15)
Helicase R	CCT CRT GGA CCG TWA TCG ACC C	2981–2960	Huang et al. (15)
Forw1_C-BLSV	GGT ATG GTT GAT TTT GCC ATA AAG	5437–5460	Bilic et al. (2)
Rev1_C-BLSV	GCT GCN CGN ARC AGT GTC GA	5717–5698	Bilic et al. (2)
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. (14)
AcGFP-13-F	TCA CAT GAA GCA GCA CGA C	843–861	Hoffmann et al. (14)
AcGFP-10-R	CTT GTA CAG CTC ATC CAT GC	1329–1310	Hoffmann et al. (14)
Probe AcGFP	HEX-TCC ACC CAG AGC GCC CTG TCC A-BHQ1	1219–1240	Hoffmann et al. (14)

^a Nucleotides that differ from the reference primer and probe sequences are written in boldface letters.^b According to EaHEV (GenBank accession number AM943646) and the sequence file of pAcGFP1-C1 (Clontech Laboratories, Inc.).

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), illustra PuReTaq Ready-To-Go PCR beads (GE Healthcare, Little Chalfont, Bucks, United Kingdom) were used with a primer concentration of 1 μ M 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, and 72°C for 1 min; and finally, 72°C for 7 min. The PCR products were run on a 2% agarose gel at 100 V for 50 min and visualized on a UV illuminator.

Generation of standard (HEV) RNA and IC RNA. For optimization of the real-time RT-PCR and generation of standard curves and the 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 the pCR4-TOPO vector using a TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA). For generation of the internal control RNA, a 712-bp PCR product (primer pair AcGFP-15-F/AcGFP-10-R; Table 2) of the green fluorescent protein (GFP) gene was amplified from the standard vector AcGFP1-C1 (Clontech Laboratories, Inc., Mountain View, CA) and cloned. Positive clones were sequenced at Eurofins MWG Operon, and clones of the correct sequence and orientation were chosen for further processing.

The vectors were linearized using PstI restriction enzyme (Invitrogen, Carlsbad, CA) and run on a 1% agarose gel for confirmation and purification. Bands of the correct size were excised and cleaned using a QIAquick gel extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The purified plasmid DNA was then *in vitro* transcribed using a MAXIscript T7 kit (Ambion, Inc., Austin, TX), according to the manufacturer's protocol, except that 1 μ l RNaseOUT (Invitrogen, Carlsbad, CA) was added to the transcription reaction mixture, and transcription was performed for 1 h at 37°C. The *in vitro*-transcribed RNA was treated by use of a Turbo DNA-free kit (Ambion, Inc., Austin, TX) 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), and the numbers of copies per μ l were calculated using the mean values and the following formula: $[(\text{g}/\mu\text{l RNA})/(\text{length} \times 340)] \times 6.022 \times 10^{23}$, where the length is the number of nucleotides.

Real-time RT-PCR. Real-time RT-PCR was performed in a 25- μ l reaction mixture on a Rotor-Gene Q apparatus (Qiagen, Hilden, Germany) using the TaqMan detection method with a QuantiTect Virus+ROX vial kit (Qiagen, Hilden, Germany). To include the internal control in the assay, in addition to the primers and probe for avian HEV, primers and probe for amplification and detection of IC RNA were included in the master mix. The final mix was named HEV-3/IC and consisted of primers BLSV-HEVf, BLSV-HEVr, AcGFP-13-F, and AcGFP-10-R, each at a final concentration of 0.4 μ M. The probes HEV-3' and AcGFP were added to the HEV-3/IC primer-probe mix at a final concentration of 0.2 μ M for each probe.

Duplex real-time RT-PCR was performed, and the yellow and green fluorescence emitted by the two probes were measured simultaneously. The conditions for RT-PCR were as follows: 50°C for 30 min, 95°C for 5 min, and three-step cycling 50 times at 95°C for 15 s, 60°C for 75 s, and 72°C for 15 s. Data analysis was done by Rotor-Gene Q software, version 1.7 (Qiagen, Hilden, Germany), by setting the threshold automatically. In order to quantify virus in samples of unknown concentration, their threshold cycle (C_T) values were compared with the standard curve and the numbers of copies of avian HEV RNA per reaction mixture were calculated. Real-time RT-PCR products were run on a 2% agarose gel to test if products of the 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.

Standard curves and internal control. For generating standard curves, 10-fold dilution series of *in vitro*-transcribed RNA from the 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 mixture. All standard curves consisted of at least five different concentrations, each of them run in duplicate.

For amplification of IC RNA, primer pair AcGFP-13-F/AcGFP-10-R, which 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 the mixture, dilution series of IC RNA were made and the smallest amount reliably detected together with HEV RNA was chosen for use in the future assay. Ultimately, the 10-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.

Real-time RT-PCR sensitivity. The lowest dilution detected by use of the standard curve was further diluted 2-fold to determine the limit of detection of *in vitro*-transcribed HEV RNA. Additionally, a sample of RNA (07/861) was serially diluted 10-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.

Real-time RT-PCR specificity. To test the specificity of the TaqMan real-time RT-PCR assay, it was performed with HEV-positive samples from swine, wild boar (genotype 3, isolate wbGER27, GenBank accession number FJ705359) (29), and 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 isolated from tissue culture). A sample of RNA isolated from the liver of a noninfected specific-pathogen-free (SPF) chicken was also tested. Internal control RNA was added to all the samples.

Nucleotide sequence accession numbers. The BLSV-HEVf/BLSV-HEVr PCR products of nine field samples were submitted to GenBank and can be found under accession numbers FN995640 to FN995648.

RESULTS

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

Nine field samples were first tested in the real-time RT-PCR with primer pair BLSV-HEVf/BLSV-HEVr and probe ORF3-HEV. It appeared that no fluorescence was detected from one sample (07/9643-2) (see below), although a band was visible by gel electrophoresis, performed to control the real-time RT-PCR (data not shown). The BLSV-HEVf/BLSV-HEVr PCR products of all nine samples were sequenced (see the data in the supplemental material), and the degenerate probe HEV-3 was designed.

Standard curves and internal control. The duplex real-time RT-PCR assay showed a detection range of over 10 orders of magnitude (2.89×10^{12} to 2.89×10^3 copies per reaction mixture). It has to be mentioned that the lowest dilution (2.89×10^3 copies per reaction mixture) 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 squared value of 0.996 were achieved (Fig. 1). The IC RNA added to the mixture used to generate the standard curve was detected reliably with an average C_T of 30.17 and a standard deviation of 0.79 (data not shown).

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 the presence of IC RNA. The standard curve obtained without IC RNA had an efficiency of 0.90 and a squared regression value of 0.9964. If IC RNA was included in the same dilution series of HEV RNA, an efficiency of 0.94 and

FIG. 1. Duplex real-time RT-PCR using primer-probe mix HEV-3/IC. Tenfold dilution series of *in vitro*-transcribed HEV RNA were spiked with equal amounts of *in vitro*-transcribed IC RNA. Every dilution was run in duplicate. (a) Amplification plots of HEV RNA; (b) standard curve obtained from HEV RNA. The efficiency was 1.04, and the regression squared value was 0.996.

a regression squared value of 0.9958 were achieved for the standard curve (Fig. 2).

Viruses in eight field samples were quantified by intercalating their C_T values to the 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. C_T values between 22.48 and 28.97 were measured, corresponding to 2.10×10^7 to 2.92×10^5 copies of avian HEV RNA per sample (Table 3).

Assay sensitivity. The concentration of 2.89×10^4 copies of *in vitro*-transcribed HEV RNA per reaction mixture was further diluted 2-fold and duplex real-time RT-PCRs were run in duplicate. All concentrations tested positive, but with the lowest dilution (1.8×10^3 copies per reaction mixture) a C_T value was obtained for only one of the two reaction mixtures. Therefore, a minimum of 3.6×10^3 copies can reliably be detected with this duplex real-time RT-PCR assay (data not shown).

RNA of sample 07/861A was diluted five times 10-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 (Fig. 3). When real-time RT-PCR was performed, the same sensitivity was determined. The undiluted sample gave almost the same C_T value as the 10^{-1} dilution (30.47 and 30.92, respectively), probably due to the presence of PCR inhibitors in the undiluted sample. For the 10^{-2} dilution, a C_T value of 33.91 was measured. The C_T of the last dilution detected (10^{-3}) was high (C_T , 40.21), indicating that the assay reached the limit of detection. For the 10^{-4} and 10^{-5} dilutions, amplification did not reach the threshold level and no C_T was measured.

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

Sample HEV RNA detection. The real-time RT-PCR assay based on the degenerate probe could detect HEV RNA in all of the 16 samples tested (Table 4). In conventional RT-PCR,

FIG. 2. Real-time RT-PCR using primer-probe mix HEV-3/IC. (a) Amplification plots of 10-fold dilution series of *in vitro*-transcribed HEV RNA with (blue lines) and without (red lines) IC RNA; (b) standard curves for 10-fold dilution series of *in vitro*-transcribed HEV RNA without IC RNA (black) and with IC (grey). The efficiencies were 0.90 and 0.94, respectively, and the regression squared values were 0.9964 and 0.9958, respectively.

TABLE 3. Samples quantified by real-time RT-PCR using primer-probe mix HEV-3/IC^a

Sample no.	C _T	No. of copies of avian HEV RNA/reaction mixture
05/2294	28.97	2.92 × 10 ⁵
05/5492	28.58	4.06 × 10 ⁵
05/6738	26.89	1.21 × 10 ⁶
05/6744-2	23.68	9.65 × 10 ⁶
05/6744-3	22.48	2.10 × 10 ⁷
05/6744-4	25.61	2.77 × 10 ⁶
05/6745-2	28.10	5.51 × 10 ⁵
08/9107	25.79	2.27 × 10 ⁶

^a All C_T values measured were in the range of the standard curve, and the number of copies of avian HEV RNA per reaction mixture was determined.

HEV RNA was detected in 9 out of 16 samples with both primer pairs (Helicase F/Helicase R and Forw1_C-BLSV/Rev1_C-BLSV). HEV RNA was detected in four additional samples with primer pair Helicase F/Helicase R but not with primer pair Forw1_C-BLSV/Rev1_C-BLSV. Neither of the two primer pairs detected HEV RNA in three samples by conventional RT-PCR (Table 4).

DISCUSSION

The RT-PCR methods for detection of avian HEV used so far are of unknown specificity for samples of different geographical origins (23). Many RT-PCRs for detection of avian

FIG. 3. Gel electrophoresis of RT-PCR products of dilution series of RNA of sample 07/861. (a) RT-PCR with primers Helicase F/Helicase R. Lane 1, 100-bp DNA ladder (Invitrogen); lanes 2 to 7, 10-fold dilutions from 10^0 to 10^{-5} ; lane 8, positive control; lanes 9 and 10, negative controls. (b) RT-PCR with primers Forw1_C-BLSV/Rev1_C-BLSV. Lane 1, 100-bp DNA ladder (Invitrogen); lanes 2 to 7, 10-fold dilutions from 10^0 to 10^{-5} ; lane 8, positive control; lanes 9 and 10, negative controls. For both primer pairs, 10^{-3} was the lowest dilution detected.

HEV RNA described in the literature use primers specially 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 that could detect RNA in liver samples from BLSV-affected birds was developed (27). Most of the RT-PCR methods for detection of avian HEV RNA are based on degenerate primer binding within ORF1 (15) and on a nested approach applying degenerate primers binding within ORF1 and ORF2 (30). These primers were developed for isolates from North America but were also successfully used for RT-PCR detection of European and Australian avian HEV (2, 21, 22, 25, 28).

Recent studies revealed that ORF3 is the most conserved region of avian HEV (2). Consequently, the TaqMan duplex real-time RT-PCR presented here uses newly developed primers and probe binding within ORF3 of the avian HEV genome and is suitable for universal detection. Additional features of real-time RT-PCR, such as quantification and use of an internal control, were integrated into the method. The design of primers and probes was based on the four available almost complete genome sequences of avian HEV isolates from Europe, Australia, and the United States (2, 5, 17). Design of an appropriate probe proved to be difficult, since probe ORF3-HEV detected HEV RNA in only 8 of 9 samples. Dually labeled probes like TaqMan can also work with a few mismatches (9), but obviously, the variability in the region of BLSV-HEVf/BLSV-HEVr was too high and so a degenerate probe was designed. In general, a degenerate probe is less specific. However, it has the advantage that it can detect different genotypes of avian HEV. Furthermore, compared to conventional PCR, probe-based detection systems such as TaqMan have improved specificity (13).

Degenerate probe HEV-3' could detect HEV RNA in 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 contained viruses mainly classified to be within genotypes 1 and 3 of avian HEV (21). One sample with virus belonging to genotype 2, according to analysis of the helicase gene sequence, was also

successfully tested with the new real-time RT-PCR method. Additionally, the two complete genome sequences of American avian HEV isolates (5, 17) were considered in primer and probe design. Therefore, the new real-time RT-PCR should also be suited for use with samples containing virus representing all the different genotypes reported so far.

In vitro-transcribed RNA was used as the 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 a standard. The method reported here detected approximately 3.6×10^3 copies of *in vitro*-transcribed HEV RNA. The published real-time PCRs for human and swine HEV (1, 8, 10, 19, 20, 26, 33) have a lower detection limit, but they use DNA as a standard, except for one assay that also used *in vitro*-transcribed RNA (1). This study used a different method for calculating the standard (number of copies of RNA) compared to the quantitative real-time RT-PCR presented here. This is probably the reason for the differences in sensitivity. The real-time RT-PCR for detection of avian HEV had a sensitivity similar to that of conventional RT-PCR using 10-fold dilutions of sample RNA. Not all HEV-positive samples were detected in conventional RT-PCR, even though two primer pairs binding within ORF1 and ORF2 were used. The real-time RT-PCR presented here has the advantage that it can detect a large range of viruses 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 virus in the samples tested in this study by use of a standard curve generated with *in vitro*-transcribed

TABLE 4. Conventional versus real-time RT-PCR using a set of field samples as templates^a

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

^a Conventional RT-PCRs were performed with two different primer 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. NA, not available; +, positive result; —, negative result.

RNA. All C_T values for the samples were in the range of the standard curve, and the amount of avian HEV present in each sample could be expressed as the number of copies of avian HEV RNA per reaction mixture. If quantification of virus is done, it must be considered that the values are theoretical and need normalization based on an equal quantity and quality of the starting material (9). The quantification of virus in 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 with various types of sample material, e.g., in studies about the pathogenicity of avian HEV.

Use of an internal control is important to exclude false-negative results due to incomplete RNA isolation or inhibition of PCR. As an alternative to synthetic fragments of RNA or DNA, housekeeping genes or different pathogens are used as an internal control. Their unknown and changing concentrations, instability, and biosafety concerns make them more difficult to handle and integrate into the PCR assay than *in vitro*-transcribed RNA (14). For these reasons, the universal heterologous internal control system designed by Hoffmann et al. (14) was chosen. Like the avian HEV genome, it is based on RNA and could easily be adapted and integrated into the assay to check for successful RNA extraction and RT-PCR. As a longer product inhibits amplification of the target template less (14), the primers for amplification of a 487-bp product were chosen (AcGFP-13-F/AcGFP-10-R). IC RNA was added in a concentration of 5.7×10^5 copies per reaction mixture, which resulted in reliable amplification of the internal control but did not inhibit amplification of avian HEV.

It is concluded that the TaqMan duplex real-time RT-PCR presented here is suitable for universal detection of avian HEVs. Besides the detection of different genotypes of avian HEV in heterologous sample material with only one primer pair, it provides essential features of real-time RT-PCR. Among these features are quantification, inclusion of an internal control, and a reduced contamination risk due to closed-tube procedures and a one-step reaction. Altogether, the real-time RT-PCR method presented in this study is a specific and sensitive diagnostic tool for universal detection of avian HEV RNA in research as well as in routine laboratory diagnostics.

ACKNOWLEDGMENTS

We thank all colleagues who provided us with tissue samples containing avian HEVs. Tissues from a wild boar and a Norway rat positive for HEVs were kindly provided by Reimar Johne, Federal Institute for Risk Assessment, Berlin, Germany. We thank Doris Zwettler und Sandra Revilla-Fernández from the Austrian Agency for Health and Food Safety (AGES), Mödling, Austria, who provided tissue material containing swine HEV.

REFERENCES

- Ahn, J. M., N. Rayamajhi, S. Gyun Kang, and H. Sang Yoo. 2006. Comparison of real-time reverse transcriptase-polymerase chain reaction and nested or commercial reverse transcriptase-polymerase chain reaction for the detection of hepatitis E virus particle in human serum. *Diagn. Microbiol. Infect. Dis.* **56**:269–274.
- Bilic, I., B. Jaskulska, A. Basic, C. J. Morrow, and M. Hess. 2009. Sequence analysis and comparison of avian hepatitis E viruses from Australia and Europe indicate the existence of different genotypes. *J. Gen. Virol.* **90**:863–873.
- Billam, P., et al. 2005. Systematic pathogenesis and replication of avian hepatitis E virus in specific-pathogen-free adult chickens. *J. Virol.* **79**:3429–3437.
- Billam, P., et al. 2008. Development and validation of a negative-strand-specific reverse transcription-PCR assay for detection of a chicken strain of hepatitis E virus: identification of nonliver replication sites. *J. Clin. Microbiol.* **46**:2630–2634.
- Billam, P., Z. F. Sun, and X. J. Meng. 2007. Analysis of the complete genomic sequence of an apparently avirulent strain of avian hepatitis E virus (avian HEV) identified major genetic differences compared with the prototype pathogenic strain of avian HEV. *J. Gen. Virol.* **88**:1538–1544.
- Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159.
- Emerson, S. U., et al. 2004. Genus Hepevirus, p. 853–857. *In* C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball (ed.), *Virus taxonomy: eighth report of the International Committee on Taxonomy of Viruses*. Elsevier/Academic Press, London, United Kingdom.
- Enouf, V., et al. 2006. Validation of single real-time TaqMan PCR assay for the detection and quantitation of four major genotypes of hepatitis E virus in clinical specimens. *J. Med. Virol.* **78**:1076–1082.
- Gunson, R. N., T. C. Collins, and W. F. Carman. 2006. Practical experience of high throughput real time PCR in the routine diagnostic virology setting. *J. Clin. Virol.* **35**:355–367.
- Gyarmati, P., et al. 2007. Universal detection of hepatitis E virus by two real-time PCR assays: TaqMan and primer-probe energy transfer. *J. Virol. Methods* **146**:226–235.
- Haqshenas, G., et al. 2002. The putative capsid protein of the newly identified avian hepatitis E virus shares antigenic epitopes with that of swine and human hepatitis E viruses and chicken big liver and spleen disease virus. *J. Gen. Virol.* **83**:2201–2209.
- Haqshenas, G., H. L. Shivaprasad, P. R. Woolcock, D. H. Read, and X. J. Meng. 2001. Genetic identification and characterization of a novel virus related to human hepatitis E virus from chickens with hepatitis-splenomegaly syndrome in the United States. *J. Gen. Virol.* **82**:2449–2462.
- Hoffmann, B., et al. 2009. A review of RT-PCR technologies used in veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health. *Vet. Microbiol.* **139**:1–23.
- Hoffmann, B., K. Depner, H. Schirrmeyer, and M. Beer. 2006. A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. *J. Virol. Methods* **136**:200–209.
- Huang, F. F., et al. 2002. Heterogeneity and seroprevalence of a newly identified avian hepatitis E virus from chickens in the United States. *J. Clin. Microbiol.* **40**:4197–4202.
- Huang, F. F., F. W. Pierson, T. E. Toth, and X. J. Meng. 2005. Construction and characterization of infectious cDNA clones of a chicken strain of hepatitis E virus (HEV), avian HEV. *J. Gen. Virol.* **86**:2585–2593.
- Huang, F. F., et al. 2004. Determination and analysis of the complete genomic sequence of avian hepatitis E virus (avian HEV) and attempts to infect rhesus monkeys with avian HEV. *J. Gen. Virol.* **85**:1609–1618.
- Johne, R., et al. 2010. Novel hepatitis E virus genotype in Norway rats, Germany. *Emerg. Infect. Dis.* **16**:1452–1455.
- Jothikumar, N., T. L. Cromeans, B. H. Robertson, X. J. Meng, and V. R. Hill. 2006. A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. *J. Virol. Methods* **131**:65–71.
- Mansuy, J. M., et al. 2004. Hepatitis E in the south west of France in individuals who have never visited an endemic area. *J. Med. Virol.* **74**:419–424.
- Marek, A., I. Bilic, I. Prokofieva, and M. Hess. 2010. Phylogenetic analysis of avian hepatitis E virus samples from European and Australian chicken flocks supports the existence of a different genus within the *Hepeviridae* comprising at least three different genotypes. *Vet. Microbiol.* **145**:54–61.
- Massi, P., et al. 2005. Big liver and spleen disease in broiler breeders in Italy. *Ital. J. Anim. Sci.* **4**:303–305.
- Meng, X. J. 2010. Hepatitis E virus: animal reservoirs and zoonotic risk. *Vet. Microbiol.* **140**:256–265.
- Meng, X. J., H. L. Shivaprasad, and C. Payne. 2008. Avian hepatitis E virus infections, p. 441–448. *In* Y. M. Saif (ed.), *Diseases of poultry*, 12th ed. Wiley-Blackwell, Ames, IA.
- Morrow, C. J., et al. 2008. Avian hepatitis E virus infection and possible associated clinical disease in broiler breeder flocks in Hungary. *Avian Pathol.* **37**:527–535.
- Orru, G., et al. 2004. Detection and quantitation of hepatitis E virus in human faeces by real-time quantitative PCR. *J. Virol. Methods* **118**:77–82.
- Payne, C. J., T. M. Ellis, S. L. Plant, A. R. Gregory, and G. E. Wilcox. 1999. Sequence data suggests big liver and spleen disease virus (BLSV) is genetically related to hepatitis E virus. *Vet. Microbiol.* **68**:119–125.
- Peralta, B., et al. 2009. Evidence of widespread infection of avian hepatitis E virus (avian HEV) in chickens from Spain. *Vet. Microbiol.* **137**:31–36.
- Schielke, A., et al. 2009. Detection of hepatitis E virus in wild boars of rural and urban regions in Germany and whole genome characterization of an endemic strain. *Virol. J.* **6**:58.
- Sun, Z. F., et al. 2004. Genetic identification of avian hepatitis E virus (HEV) from healthy chicken flocks and characterization of the capsid gene of 14 avian HEV isolates from chickens with hepatitis-splenomegaly syndrome in

- different geographical regions of the United States. *J. Gen. Virol.* **85**:693–700.
31. **Sun, Z. F., et al.** 2004. Generation and infectivity titration of an infectious stock of avian hepatitis E virus (HEV) in chickens and cross-species infection of turkeys with avian HEV. *J. Clin. Microbiol.* **42**:2658–2662.
32. **Todd, D., K. A. Mawhinney, V. A. McAlinden, and A. J. Douglas.** 1993. Development of an enzyme-linked immunosorbent assay for the serological diagnosis of big liver and spleen disease. *Avian Dis.* **37**:811–816.
33. **Ward, P., et al.** 2009. Comparative analysis of different TaqMan real-time RT-PCR assays for the detection of swine hepatitis E virus and integration of feline calicivirus as internal control. *J. Appl. Microbiol.* **106**:1360–1369.