Applied and Environmental Microbiology, Apr. 2010, p. 2165–2174 Vol. 76, No. 7
0099-2240/10/$12.00 doi:10.1128/AEM.01929-09

Detection and Quantification of Infectious Avian Influenza A (H5N1) Virus in Environmental Water by Using Real-Time Reverse Transcription-PCR▽

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Received 12 August 2009/Accepted 25 January 2010

Routes of avian influenza virus (AIV) dispersal among aquatic birds involve direct (bird-to-bird) and indirect (waterborne) transmission. The environmental persistence of H5N1 virus in natural water reservoirs can be assessed by isolation of virus in embryonated chicken eggs. Here we describe development and evaluation of a real-time quantitative reverse transcription (RT)-PCR (qRT-PCR) method for detection of H5N1 AIV in environmental water. This method is based on adsorption of virus particles to formalin-fixed erythrocytes, followed by qRT-PCR detection. The numbers of hemagglutinin RNA copies from H5N1 highly pathogenic AIV particles adsorbed to erythrocytes detected correlated highly with the infectious doses of the virus that were determined for three different types of artificially inoculated environmental water over a 17-day incubation period. The advantages of this method include detection and quantification of infectious H5N1 AIVs with a high level of sensitivity, a wide dynamic range, and reproducibility, as well as increased biosecurity. The lowest concentration of H5N1 virus that could be reproducibly detected was 0.91 50% egg infective dose per ml. In addition, a virus with high virion stability (*Tobacco mosaic virus*) was used as an internal control to accurately monitor the efficiency of RNA purification, cDNA synthesis, and PCR amplification for each individual sample. This detection system could be useful for rapid high-throughput monitoring for the presence of H5N1 AIVs in environmental water and in studies designed to explore the viability and epidemiology of these viruses in different waterfowl ecosystems. The proposed method may also be adapted for detection of other AIVs and for assessment of their prevalence and distribution in environmental reservoirs.

Wild aquatic birds belonging to the orders Anseriformes (ducks, geese, swans) and Charadriiformes (gulls, terns, shore birds) are the natural reservoirs of avian influenza viruses (AIVs) and play an important role in their ecology and propagation (30, 37). The epidemiology of AIVs is best understood in ducks, for which predictable temporal and spatial patterns of infection have been reported (29). Most AIVs replicate preferentially in the gastrointestinal tract of ducks, high concentrations are excreted in feces, and the viruses are transmitted via the fecal-oral route, sometimes involving contaminated water in shared aquatic habitats (11).

The transmission and persistence of AIVs in wild bird populations remain poorly understood because they depend both on the ecology of the host (e.g., population density and migration patterns) and on the environment (e.g., water characteristics may influence AIV persistence). The dispersal of AIVs among aquatic birds involves direct (bird-to-bird) and indirect (waterborne) transmission (24). Indeed, these viruses can be transmitted without direct contact between birds, and this can provide a natural mechanism for maintaining influenza viruses in avian species (4, 5, 14, 31, 32). This waterborne indirect transmission may play an important role in the epidemiology of influenza virus infections or may even be the main determinant of the disease dynamics in wild birds, highlighting the potential importance of the persistence of viral particles in water (24, 26). However, the importance of waterborne transmission in natural ecosystems has not been adequately evaluated, especially for the emerged H5N1 highly pathogenic avian influenza viruses (HPAIVs). In contrast to wild-type AIVs, greater titers of the H5N1 HPAIVs are shed for longer durations from the trachea and upper respiratory tract than by the cloacal route (2, 34). Although it is possible that H5N1 respiratory infections do not result in the same levels of environmental contamination as fecal shedding, this factor may be offset by the possibility that transmission of H5N1 may require a low infective dose (3).

Highly pathogenic H5N1 viruses have been responsible for outbreaks in poultry over the last 13 years, resulting in the longest recorded HPAIV circulation in poultry. Due to its continuous circulation during this period, this virus lineage has undergone extensive genotype alterations through reassortment, which produced numerous H5N1 genotypes that evolved into 10 distinct H5 virus hemagglutinin (HA) lineages, as de-

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▽ Published ahead of print on 29 January 2010.

TABLE 1. Specific primers and probes used in real-time PCR for detection of the avian influenza virus H5 gene, *Tobacco mosaic virus*, and the influenza virus A matrix gene

Specificity	Primer or probe	Sequence (5′→3′)[a]	Location[b]	Concn in PCR mixture (nM)	Estimated melting temp (°C)[c]
Avian H5 virus gene	H5-F1532	ACGTATGACTAYCCGCAGTATTCAGAAGA	1532–1560	400	67.9–69.3
	H5-P1646	FAM-ATGATTGCCAGTGCTAGRGARCTCGC-BHQ-1	1671–1646	300	69.9–71.5
	H5-R1692	AACGAYCCATTGGAGCACATCC	1713–1692	400	66.2–68.1
TMV	TMV-F4675	AGTTGATCTCGAAACTTGGTGCT	4675–4697	100	65.0
	TMV-P4713	HEX-TGGGAACACTTGGAGGAGTTCAGAAGGTCT-BHQ-1	4713–4742	150	71.8
	TMV-R4802	AGCGTCGTCCAACTGTGTGT	4783–4802	100	66.6
Influenza virus A matrix gene	InA/M-F	AGACCRATCYTGTCACCTCTGAC	170–192	800	65.2–67.9
	InA/M-P	FAM-TCACCGTGCCCAGTGAGCGAGGACTGC-BHQ-1	222–248	200	76.1
	InA/M-R	AGGGCATTYTGRACAAAICGTCTACG	251–276	800	67.2–69.2

[a] IUPAC ambiguity codes are used (R = A or G; Y = C or T). The most frequently encountered mismatches with the targeted sequences are indicated by underlining.
[b] Primer and probe annealing positions corresponding to positions in the HA or matrix gene of A/mute swan/Germany/R1349/07 (H5N1) (accession numbers AM749442 and AM913994, respectively) and to positions in the TMV genome (based on accession number AF165190).
[c] The melting temperatures were estimated using the OligoAnalyzer 3.1 software (Integrated DNA Technologies).

scribed by the World Health Organization/World Organization for Animal Health/Food and Agriculture Organization H5N1 Evolution Working Group (38). The ecological success of this virus in many avian species allowed geographical expansion worldwide, which necessitated intense virological monitoring of wild bird populations (8, 12, 19, 20, 33). Although H5N1 AIVs can persist for extended periods of time in water at wide ranges of temperature and salinity (4), the impact of environmental contamination on the transmission between birds and the persistence of the infection in aquatic habitats are not known.

The concentrations of influenza viruses excreted by infected birds in natural water reservoirs are often below the threshold of detection of most commonly used diagnostic methods. The most sensitive detection methods are virus isolation in embryonated specific-pathogen-free (SPF) chicken eggs and reverse transcription (RT)-PCR (15). Isolation in SPF eggs is highly sensitive for detection of infectious virus particles. However, this method is quite labor-intensive and time-consuming, and, especially in the case of H5N1, it requires maintenance of a high biosecurity level in order to prevent exposure of humans to the virus because it involves replication of the virus to produce high titers. On the other hand, real-time RT-PCR is a sensitive and reliable method for detection and quantification of AIVs and does not involve replication of infectious virus. However, a correlation between the number of virus RNA copies detected by RT-PCR and virus infectivity in samples has not been reported.

In this study we describe development of a novel real-time quantitative assay for the H5 virus hemagglutinin (HA) gene, which is fully controlled in order to monitor the efficiency of RNA purification, cDNA synthesis, PCR amplification, and signal detection for each individual sample. The method described here can detect the virus in large quantities of water and can quantify infectious viruses. The methodology is based on virus adsorption onto formalin-fixed erythrocytes, followed by detection and quantification by real-time RT-PCR. The main advantages of this method are its high sensitivity for detection of infectious virus and lower risk to operators as no replication of the virus is involved.

MATERIALS AND METHODS

H5N1 HPAIV isolates, virus propagation, and titration. Fourteen H5N1 HPAIV isolates were used in the experiments described here. All isolates were recovered from affected birds during the virus incursion in Greece in February and March 2006. Two isolates, A/red-breasted goose/Greece/167/06 from Skyros Island and A/mute swan/Greece/436/06 from the Veria region, were propagated in 10-day-old SPF embryonated chicken eggs (ECEs). Infective amnio-allantoic fluid (AAF) was harvested at 96 h postinoculation and titrated to determine the 50% egg infective dose (EID_{50})/ml using ECEs (36) and to determine the 50% tissue culture infective dose ($TCID_{50}$)/ml using cell suspensions prepared as primary cultures of chicken embryo fibroblasts (CEFs) (4). The virus titer was calculated by the Reed-Muench method (22). Eleven isolates (188/06, 222/06, 230/06, 262/06, 266/06, 288/06, 346/06, 350/06, 432/06, 435/06, and 492/06) recovered from 11 swans and one isolate (391/06) recovered from a cormorant were propagated in ECEs, and the AAF was tested using the real-time RT-PCR described below. All experiments in which material containing infectious HPAIV was handled were conducted in biosafety level 3 (BSL-3) facilities at the Faculty of Veterinary Medicine, Aristotle University of Thessaloniki.

Adsorption of AIV diluted with water to formalin-fixed chicken erythrocytes. Twenty-four microliters from a 50 mM stock solution of a neuraminidase inhibitor (4-guanidino-Neu5Ac2en) and 200 μl from a 10% (vol/vol) suspension of formalin-fixed chicken red blood cells (CRBCs) were added to 50 ml of either environmental water or sterile double-distilled water (ddw) inoculated with infective AAF. The 4-guanidino-Neu5Ac2en stock solutions were prepared using Relenza (GlaxoSmithKline, Evreux, France) Rotadisks (5 mg of zanamivir with lactose) by dissolving 5-mg blister capsules in 285 μl of phosphate-buffered saline (PBS) and were stored at −20°C. The CRBCs were formalin fixed as previously described (15). After fixation, the erythrocyte solution was washed eight times, and it could be stored for up to 6 months at 4°C in PBS as a 10% (vol/vol) suspension containing antibiotics and antimycotics at the following final concentrations: 200 U/ml penicillin G, 200 μg/ml streptomycin sulfate, and 0.5 μg/ml amphotericin. The tubes containing AIV-inoculated water with CRBCs were mixed constantly at room temperature using a rotator at 2 rpm for 90 min to allow adsorption of virus to CRBCs. The CRBCs with the adsorbed virus were pelleted by centrifugation (20 min at 1,800 × *g*). The pellet was resuspended in 1.5 ml PBS, transferred to a 2-ml tube, and pelleted again by centrifugation (3 min at 6,000 × *g*). The CRBCs were washed again with 1.5 ml PBS in the same tube, and finally the pellet was resuspended in ddw (final volume, 140 μl). RNA extraction and quantitative RT-PCR (qRT-PCR) were then preformed as described below.

Preparation of H5 HA RNA standard and TMV internal control. *In vitro*-transcribed H5 RNA was prepared as previously described (23). Briefly, a 557-bp HA amplicon was produced by RT-PCR using RNA extracted from A/mute swan/Greece/436/06 and primers H5-F1157 (GGGAGTGGGTACGCTGC AGA) and H5-R1692 (Table 1). The amplicon was cloned into pGEM-T Easy vector system I (Promega, Mannheim, Germany). *In vitro* transcription reactions were performed using 1 μg linearized plasmid and T7 RNA polymerase (MBI Fermentas, St. Leon-Rot, Germany). The mixture was treated with 5 U RNase-

free DNase I (Ambion, Austin, TX). *In vitro* transcripts were recovered by phenol-chloroform extraction and ethanol precipitation and were quantified by spectrophotometry. RNA was also extracted from allantoic fluid containing each of the two viruses (A/red-breasted-goose/Greece/167/06 and A/mute swan/Greece/436/06) with known virus titers. Dilutions of these RNA preparations along with titrated virus stocks from allantoic fluid were used to determine the amplification efficiency, variability, dynamic range of quantification, and detection limit of the qRT-PCR assays. *Tobacco mosaic virus* (TMV) was purified from infected *Nicotiana tabacum* plants by two cycles of differential centrifugation and precipitation with polyethylene glycol (PEG) using the method of the Association of Applied Biologists for purification of *Tomato mosaic virus* (http://www.dpvweb.net/dpv/showdpv.php?dpvno=156) based on the method of Gooding and Hebert (10). The RNA titer of the purified TMV suspension was estimated by Poisson endpoint dilution analysis (25) using the optimized TMV-specific real-time RT-PCR described below. The suspension was diluted with 10 mM phosphate buffer (pH 7.4) to obtain a solution containing approximately 1.5×10^6 copies of TMV RNA/μl and was stored in aliquots at −70°C.

RNA extraction. A QIAamp viral RNA minikit (Qiagen, Hilden, Germany) was used to extract total RNA directly from 140-μl virus-infected water samples or from CRBCs with adsorbed virus particles. In the latter case, 140 μl of a resuspended CRBC mixture was directly added to AVL carrier RNA buffer, incubated for 10 min at room temperature, and then clarified by centrifugation at 12,000 × *g* for 3 min. Subsequently the manufacturer's protocol was followed, except that it was modified by addition of a second wash with 300 μl AW2 buffer. RNA was eluted in 60 μl of AVE buffer. In order to minimize the risk of generating false-negative results and false low values, every sample was spiked with TMV particles. For every sample 0.2 μl of purified TMV particles was added to the AVL buffer at the extraction stage, which was equivalent to addition of approximately 3×10^5 copies of TMV RNA.

Design of primers and hydrolysis probes. All available different genotypes of complete HA nucleotide sequences from H5N1 isolates determined since 2005 were downloaded from the NCBI Influenza Virus Resource Database (http://www.ncbi.nlm.nih.gov/genomes/FLU) and aligned (clustal X, version 2.0). The final data set was comprised of 343 European or African and 697 Asian HA sequences. An influenza virus H5 gene-specific PCR primer set and a hydrolysis probe were designed using conserved genomic regions tolerating one or two degenerate sites per oligonucleotide in order to accommodate the variability revealed by the alignment. The primers and probe were designed so that each oligonucleotide shared over 96% identity with the targeted HA sequences, allowing only a one-base mismatch with a small percentage of the sequences. In cases where two mismatches had to be tolerated, the mismatches were located in the 5′ terminus of the oligonucleotide. Successful binding of the primers and probe when there were target mismatches was assessed by calculating the base mismatch DNA thermodynamics using the OligoAnalyzer 3.1 software developed by IDT (Integrated DNA Technologies, Coralville, IA) and the DINAMelt web server (http://dinamelt.bioinfo.rpi.edu/refs.php) (18). The sequences and details concerning primers and probes are shown in Table 1. Similarly, two specific primers and a TaqMan probe were designed using highly conserved regions of the RNA-dependent RNA polymerase (RdRp) gene of *Tobacco mosaic virus*, which was used in the assay as an internal control (accession no. AF165190). The H5N1- and TMV-specific TaqMan probes were labeled at the 5′ end with the 6-carboxyfluoresin (FAM) and hexachloro-carbonylfluorescein (HEX) reporter dyes, respectively, and labeled at the 3′ end with the quenching dye black hole quencher 1 (BHQ-1). Influenza virus matrix-specific primers (Table 1) were designed using a data set obtained from 611 full-length nonidentical matrix sequences from all available environmental and animal isolates, which was retrieved from the Influenza Virus Resource at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html).

qRT-PCR. The reverse transcription (RT) reaction mixture (final volume, 20 μl) contained 1× first-strand buffer, 0.01 M dithiothreitol (DTT), each deoxynucleoside triphosphate (dNTP) at a concentration of 0.5 mM, 1.4 μM random hexamers, 50 U Moloney murine leukemia virus (M-MLV) reverse transcriptase, 13 U RNase inhibitor, 12 μl of sample RNA, and RNase-free water (all reagents were obtained from Invitrogen, the Netherlands). This reaction mixture was then incubated under the following thermal conditions: 25°C for 10 min, 37°C for 40 min, 42°C for 10 min, and 95°C for 10 min. Then it was cooled to 4°C. Five microliters of cDNA was added to a separate 50-μl real-time qPCR mixture. The PCRs were optimized for standard cycling conditions using an initial denaturation step of 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 30 s and annealing at 60°C for 1 min, using the Mx3005P QPCR system (Stratagene, La Jolla, CA). The optimal multiplex reaction conditions for qPCR were as follows: 2.5 U of Platinum *Taq* DNA polymerase (Invitrogen, the Netherlands), 5 μl 10× PCR buffer, 400 μM each of dATP, dCTP, dGTP, and dTTP, 6 mM $MgCl_2$, H5 HA- and TMV-specific primers and probes at the concentrations shown in Table 1, and nuclease-free water (final volume, 50 μl). For each dye, the fluorescence data analysis was conducted using the MxPro-Mx3005P software (version 4.00; Stratagene, La Jolla, CA), the option for baseline correction was an adaptive baseline, and the threshold fluorescence levels used to derive cycle threshold (C_T) values were determined automatically. The TMV C_T value for each sample was required to be within 2 standard deviations of the mean of the TMV C_T values. Samples with TMV C_T values that deviated from the mean by more than 2 standard deviations were retested. To generate a standard curve for correlation of C_T values with virus infectivity and to evaluate the inter- and intra-assay variability of the qRT-PCR employed, different log dilutions of a known titer ($TCID_{50}$/ml and ECD_{50}/ml) of H5N1 virus in ddw were prepared and tested using qRT-PCR. RNA was extracted either directly from the inoculated water or from H5N1 AIV particles after adsorption to CRBCs.

The specificity of the H5 HA-specific primers and probe in the multiplex real-time RT-PCR assay was also evaluated by testing RNA extracts of isolates belonging to different influenza subtypes (Table 2). An influenza A matrix-specific RT-PCR assay was used to verify that all influenza virus isolates were PCR positive. Here, the matrix-specific primers and probe were used at concentrations shown in Table 1 by using the cycling and reaction conditions described for the multiplex real-time PCR above.

Sequencing. To confirm the specificity of amplification, PCR products were purified and sequenced using a BigDye Terminator v3.0 ready reaction cycler sequencer kit (Applied Biosystems) according to the manufacturer's instructions.

Water experiment: comparison of virus titration using CEFs and qRT-PCR. To compare microtiter endpoint titration and qRT-PCR results, environmental water samples were inoculated with H5N1 virus and subsequently tested in parallel using CEFs and qRT-PCR assays over a 17-day period. Samples of environmental water were collected from three different areas in the Evros River delta wetland in northeastern Greece. The collection sites were areas where waterfowl are often observed (feeding, roosting, swimming, etc.), and there is variation in habitats, salinity, and pH among these sites. The water samples were transported at 4°C, clarified by centrifugation (5 min at 3,000 × *g*), and sterilized by passage through a 0.22-μm filter. All three types of water (type a, river water; type b, lagoon water; and type c, stable brackish water from the bank of a flooded zone) along with ddw were inoculated with A/mute swan/Greece/436/06 (H5N1) AIV to obtain final concentrations of $10^{3.7}$ to $10^{5.7}$ $TCID_{50}$/ml. Two-milliliter aliquots of each inoculated type of water were kept at 4°C. Starting on day 0 postinoculation, aliquots were titrated twice a week using CEFs and qRT-PCR. Quantification of the total H5 HA gene copies was performed using RNA directly extracted from 140 μl of inoculated water and qRT-PCR. Infectivity ($TCID_{50}$/ml) was quantified by microtiter endpoint titration using CEFs beginning with inoculated water diluted 1:2 with minimal essential medium. Quantification of infectious virus by qRT-PCR was performed after dilution of 140 μl of inoculated water with 50 ml of the same type of water, followed by treatment

TABLE 2. Influenza virus strains examined by H5 virus- or matrix-specific real-time RT-PCR in a specificity test

Influenza virus strain	Subtype	Test results	
		Matrix RT-PCR	H5 virus RT-PCR
A/Thessaloniki/1322/2009v[a]	H1N1	+	−
A/dk/Alb/35/76[b]	H1N1	+	−
A/dk/Germ/1215/73[b]	H2N3	+	−
A/tky/England/69[b]	H3N2	+	−
A/dk/Czech/58[b]	H4N6	+	−
A/ck/Scot/59[b]	H5N1	+	+
A/Northern Shoveler/Greece/892/06[c]	H6N8	+	−
A/Afr starling/England/983/79[b]	H7N1	+	−
A/tky/Ontario/6118/68[b]	H8N4	+	−
A/tky/Wisc/1/66[b]	H9N2	+	−

[a] Novel 2009 influenza A (H1N1)v RNA from a human clinical sample obtained by the National Influenza Centre for North Greece, B′ Laboratory of Microbiology, School of Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece.

[b] AIV purchased from the Veterinary Laboratories Agency New Haw, Addlestone, Surrey, United Kingdom.

[c] AIV isolated by the CTVI/IIPD, Department of Avian Diseases, Thessaloniki, Greece.

with formalin-fixed CRBCs. The corresponding titers determined by qRT-PCR were multiplied by the dilution factor (357) for final comparisons with the titers obtained for the undiluted samples using CEFs.

Statistical analysis. Probit regression was used to model the relationship between the number of RNA copies present in the RT reaction and a positive test result obtained by qRT-PCR. For this purpose, eight replicates were tested at each of the following four concentrations: 108, 72, 48, and 24 RNA copies per RT reaction mixture. Using the resulting model, we estimated the 95% detection limit of the method. Furthermore, the relationship between the logarithm of titer results obtained by qRT-PCR using CRBCs and microtiter endpoint titration was assessed using simple linear regression. Linear regression models were also used to assess whether the decline in the logarithm of virus titers, as determined using qRT-PCR with CRBCs over time, was influenced by the type of environmental water that was used. Two linear regression models were fitted, one for each initial virus concentration. In each model, the dependent variable was log virus titer determined by qRT-PCR with CRBCs in all three types of environmental water. The independent variables were time and two indicator (0 and 1) variables describing the three levels of the variable "environmental water used" (type a water, river water; type b water, lagoon water; and type c water, brackish water). The model also included the corresponding two-way interactions between the time and type of water variables. Therefore, for each initial virus concentration a linear regression model was fitted, with the following response function: $E(\log_{10}\text{ virus titer}) = \alpha + \beta_1(\text{time}) + \beta_2(\text{water}_1) + \beta_3(\text{water}_2) + \beta_4(\text{water}_1 \times \text{time}) + \beta_5(\text{water}_2 \times \text{time})$. In this model, the two indicator variables, (water_1) and (water_2), describe the three levels of the variable "environmental water used." Specifically, each of these two variables can have the value 0 or 1. Using this scheme, "water type a" could be described as $(\text{water}_1) = 0$ and $(\text{water}_2) = 0$; "water type b" could be described as $(\text{water}_1) = 1$ and $(\text{water}_2) = 0$; and "water type c" could be described as $(\text{water}_1) = 0$ and $(\text{water}_2) = 1$. In this way, the regression model outlined above can describe three regression lines, one for each type of water, as follows. For "water type a," $(\text{water}_1) = 0$ and $(\text{water}_2) = 0$, and the response function is $E(\log_{10}\text{ virus titer}) = \alpha + \beta_1(\text{time})$. For "water type b," $(\text{water}_1) = 1$ and $(\text{water}_2) = 0$, and the response function is $E(\log_{10}\text{ virus titer}) = \alpha + \beta_1(\text{time}) + \beta_2 + \beta_4(\text{time}) = (\alpha + \beta_2) + (\beta_1 + \beta_4)(\text{time})$. For "water type c," $(\text{water}_1) = 0$ and $(\text{water}_2) = 1$, and the response function is $E(\log_{10}\text{ virus titer}) = \alpha + \beta_1(\text{time}) + \beta_3 + \beta_5(\text{time}) = (\alpha + \beta_3) + (\beta_1 + \beta_5)(\text{time})$.

By assessing the significance of appropriate regression coefficients or groups of regression coefficients, we could assess whether the three lines (for decline in virus infectivity over time measured by the qRT-PCR assay using CRBCs in each of the three types of water) could be considered to be coincident, parallel, or neither coincident nor parallel. A similar approach was used to model the decline in the logarithm of virus titers determined both by qRT-PCR using CRBCs and by microtiter endpoint titration separately for each combination of type of water and initial virus concentration. Six multiple linear regression models were fitted, one for each combination of initial virus concentration and type of water. The dependent variable in each of these models was log virus titer, while the independent variables were time and type of test (0 = qRT-PCR with CRBCs; 1 = microtiter endpoint titration). The interaction between time and test was included in the model. Based on the significance of appropriate regression coefficients or groups of regression coefficients, in each of the six models we could assess whether the two lines (for the decline in virus infectivity over time measured by each of the two assays) could be considered coincident or parallel or neither for every possible combination of initial virus concentration and water. Whenever statistical significance was assessed, the level of significance was 0.05. Statistical analyses were conducted using the STATA software (Stata Statistical Software, release 10, 2007; StataCorp. LP, College Station, TX).

RESULTS

Amplification efficiency, dynamic range, and sensitivity of the qRT-PCR. All qRT-PCR assays that were performed with serially diluted *in vitro*-transcribed RNA and the viral RNA from the two H5N1 virus isolates resulted in amplification plots with the same calibration curve slopes showing the same amplification efficiency. The linear range of detection was from 2×10^8 to 72 RNA copies per reverse transcription reaction, with an amplification efficiency of 99.9%. A typical amplification plot for a 10-fold dilution series of H5 transcribed RNA used to construct the standard curve is shown in Fig. 1A. The amplification efficiency and linearity of the standard curve for dilutions of low HA RNA concentrations were not affected by the presence of the TMV RNA internal control in each reaction mixture (Fig. 1). The 95% detection limit, as estimated by probit regression modeling, was 61.6 RNA copies per RT. When the experiment was repeated without the TMV primers and probe in the qRT-PCR, the lower detection limit of the assay was not affected. The C_T values obtained from qRT-PCR using H5N1 HPAIV particles adsorbed to CRBCs from 50-ml water samples were associated with the titers in water expressed as TCID_{50}/ml and EID_{50}/ml with CEFs and ECEs, respectively. A linear dynamic quantification range of approximately 5 $\log_{10}$ (0.91 to 10^5 EID_{50}/ml or 0.057 to $10^{3.81}$ TCID_{50}/ml) was observed (Fig. 2). The lowest concentration of H5N1 virus (expressed as the infective dose/ml) in a water sample that could be reproducibly detected was 0.91 EID_{50}/ml or 0.057 TCID_{50}/ml. The estimated ratio of the number of infectious particles (calculated as EID_{50}) to the number of HA RNA copies detected from AIV particles adsorbed to CRBCs was 1:17.4.

Specificity. All 14 H5N1 isolates examined using the H5-specific oligonucleotides were detected successfully. Amplicons derived from virus isolates 167 and 436 were sequenced, and both sequences were confirmed to be sequences of the H5 HA gene exhibiting 100% identity with the TaqMan probe. All available full and partial HA sequences that included the PCR-targeted region were retrieved and aligned using the GISAID/EpiFluDB platform (http://platform.gisaid.org/). The data sets comprised 604 European or African and 891 Asian H5N1 viruses isolated since 2005. Sequence comparisons of the targeted regions indicated that oligonucleotides H5-F1532, H5-R1692, and H5-P1646 were 100% complementary to high percentages of European or African (95.7%, 93.7%, and 84.3%, respectively) and Asian (72.5%, 85.6%, and 81.9%, respectively) HA sequences from H5N1 AIVs circulating after 2005. The most frequently encountered mismatches with the targeted sequences are indicated in Table 1. Oligonucleotides H5-R1692 and H5-P1646 each had only one base mismatch with the sequences that were not 100% complementary. In the case of primer H5-F1532 two mismatches located in the 5′ terminus were observed for 11.1% of the Asian sequences. All of these sequences were from Indonesia and belonged to subclade 2.1.3, as described by the World Health Organization/World Organization for Animal Health/Food and Agriculture Organization H5N1 Evolution Working Group (38). All three oligonucleotides were designed to have high melting temperatures (Table 1). Calculated base mismatch DNA thermodynamics suggested that the single mismatches identified are not adequate to prevent successful binding of these oligonucleotides under the annealing conditions used for the real-time PCR described in Materials and Methods (data not shown).

Comparison of sequence homology using BLAST searches (1) predicted that the H5 and TMV primer-probe sets would be specific for their corresponding targets and would not cross-react with different influenza virus HA subtypes or other microbial sequences. To further assess the specificity of the methods, AIV-negative swabs (tracheal and cloacal) from 20 uninfected wild birds collected in Greece during 2009 and 10 random samples of 50 ml environmental water treated with CRBCs were tested, and all of the results were negative. All these samples were verified to be negative by isolation of virus

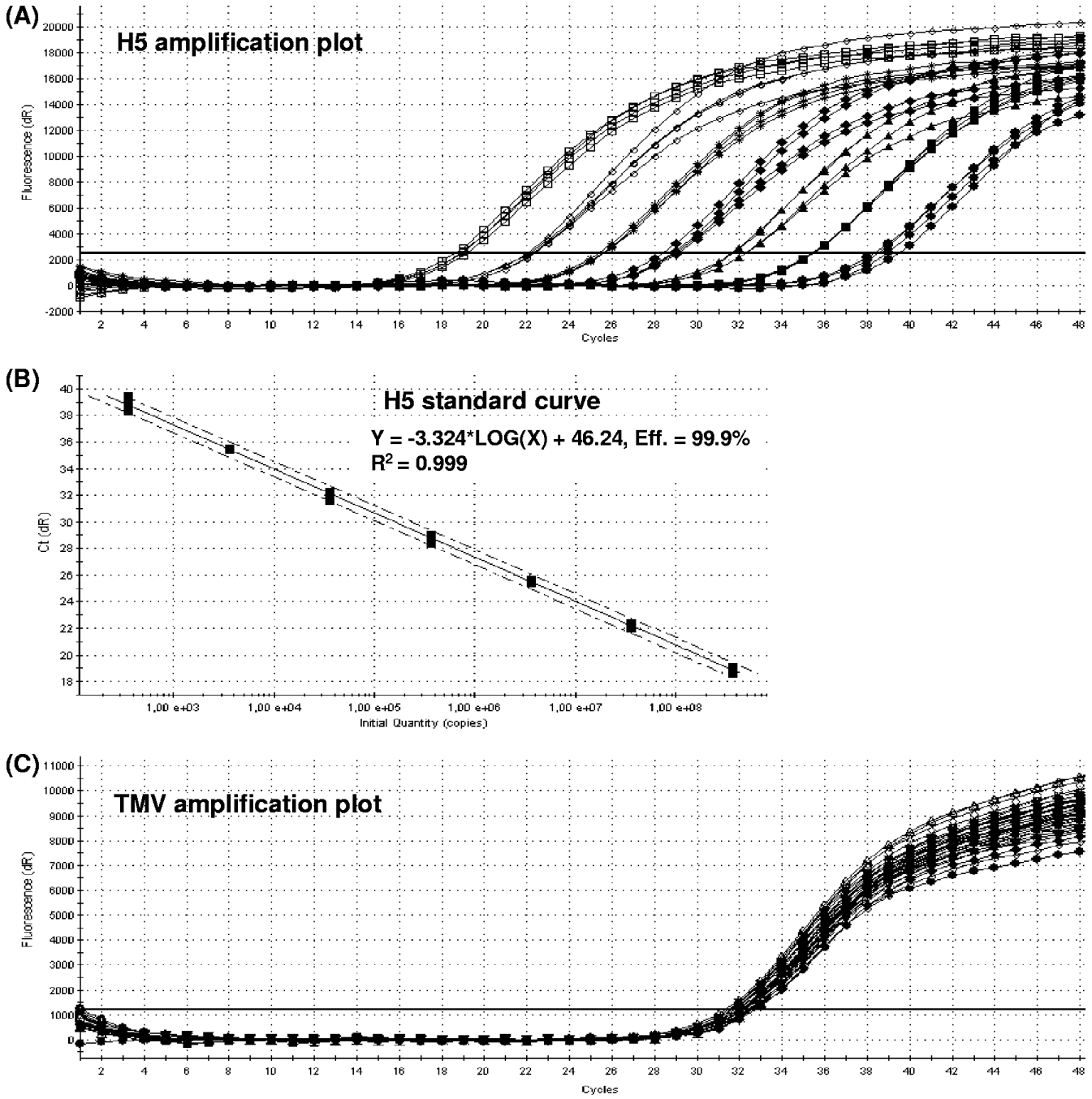

FIG. 1. Amplification plots for H5 HA RNA and TMV detection. (A and B) FAM fluorescent signals (A) and corresponding standard curve generated from a 10-fold dilution series (B) of H5 HA RNA (the 95% confidence limits for the standard curve are indicated by dashed lines in panel B). From left to right, the curves in panel A show results for RNA concentrations ranging from 2×10^8 to 200 copies per reverse transcription reaction performed with four replicates. (C) HEX fluorescent signals generated from the internal TMV control, demonstrating that all samples had a satisfactory TMV C_T (i.e., a C_T within 2 standard deviations of the mean), irrespective of the concentration of the HA RNA target.

from ECEs. Additional evidence showing the specificity of the H5 HA-specific primers and probe was obtained after examination of different influenza virus subtypes, including the H2, H1, and H6 subtypes, which are the subtypes most closely related genetically to subtype H5. None of the different subtypes, including the novel 2009 influenza A (H1N1) virus from a human clinical sample, produced a fluorescent signal in the multiplex real-time RT-PCR (Table 1). The data for all influenza virus subtypes were confirmed by the matrix-specific real-time RT-PCR.

Reproducibility. The interassay coefficient of variation (based on the calculated HA RNA concentration expressed in number of copies per ml of water) was calculated from the results of eight consecutive qRT-PCR assays performed over a 2-week

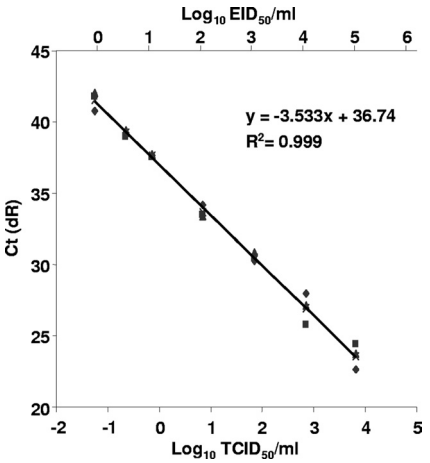

FIG. 2. Analysis of the qRT-PCR linearity using CRBC-adsorbed virus particles from 50-ml samples of serially diluted distilled water inoculated with an infectious dose of H5N1 HPAIV. The standard curve was generated from C_T values plotted against the corresponding titers expressed as the 50% tissue culture infective doses ($TCID_{50}$)/ml or the 50% egg infective doses (EID_{50})/ml.

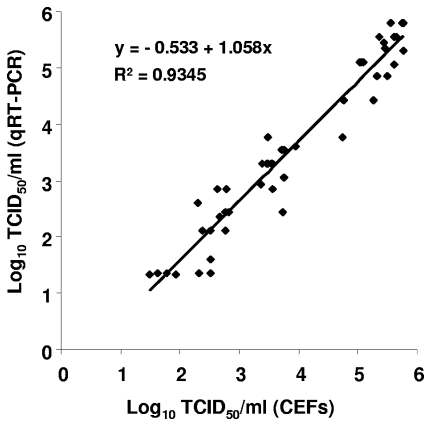

FIG. 3. Linear regression line for the quantified infectivity of H5N1 HPAIV in artificially inoculated water samples tested over time, as determined using chicken embryo fibroblasts (CEFs) and qRT-PCR with virus particles adsorbed to CRBCs. In both cases titers are expressed in log 50% tissue culture infective doses ($TCID_{50}$)/ml.

period, using ddw samples artificially inoculated with H5N1 virus and stored at 4°C. The samples were extracted either directly or after treatment with CRBCs. Similarly, the intra-assay coefficient of variation was calculated from the results of six qRT-PCR assays. The experimental intra- and interassay variability values obtained using different virus loads are summarized in Table 3.

Performance using inoculated environmental water samples. To determine if there was a correlation between the quantitative results of the qRT-PCR assays and the tissue culture titration results, a qRT-PCR assay using CRBCs and a microtiter endpoint titration assay were carried out using the same samples from three different types of environmental water and ddw inoculated with H5N1. Linear regression analysis of the logarithm of $TCID_{50}$ values for all samples tested over a 17-day period after inoculation, determined by microtiter endpoint titration and the qRT-PCR assay using CRBCs, showed that there was a significant linear relationship between the two sets of values; the 95% confidence interval for the beta coefficient was to 0.975 and 1.141, and R^2 was 0.935 (Fig. 3). The fact that the confidence interval includes 1 shows that the null hypothesis for the regression coefficient with a β value of 1 could not be rejected at the 5% level of significance. This test was equivalent to testing the possibility that the slope of the regression line is 45°.

Figure 4 shows that there was a substantial decrease in virus infectivity over time for the three types of environmental water, which was observed using both methods (microtiter endpoint titration and qRT-PCR using CRBCs). In contrast, no apparent decrease in the total number of HA copies over time was detected when quantification was performed by qRT-PCR without CRBCs. In ddw, the virus infectivity, as determined by any of the three testing methods, remained relatively constant during the 17-day period.

Linear regression modeling was used to compare the rates of decline of virus infectivity by qRT-PCR using CRBCs over time for the three different types of environmental water that were tested. For an initial virus concentration of $10^{5.7}$ $TCID_{50}$/ml the regression lines describing the decrease in virus titers in the three types of water could not be considered either coincident or parallel. However, water type a and water type c could be modeled by one regression line, while the regression line for water type b had the same intercept but a different (lower absolute value) slope. For an initial virus concentration of $10^{3.7}$ $TCID_{50}$/ml the regression lines describing the decreases in virus titers in the three types of water could also not be considered either coincident or parallel. However, water type a and water type b could be modeled by one regression line, while the regression line for water type c had a different intercept and a different slope.

Linear regression analysis of the decrease in log virus titers, as determined by microtiter endpoint titration and qRT-PCR using CRBCs, showed that the two regression lines could be considered coincident in all six fitted regression models (i.e., for all six combinations of initial virus concentration and type of water).

The mean C_T for the TMV internal control of each real-time PCR run ranged from 32.12 to 33.38 cycles for all water sam-

TABLE 3. Inter- and intra-assay variability of the qRT-PCR method based on calculated HA RNA concentrations with or without application of formalin-fixed chicken red blood cells

Variation	No. of RNA copies/ml of water	Coefficient of variation (%)	
		qRT-PCR without CRBCs	qRT-PCR with CRBCs
Intra-assay	1×10^9	13.42	NT[a]
	1×10^8	18.16	NT
	1×10^7	17.02	18.99
	1×10^6	9.27	15.67
	1×10^5	16.58	30.02
	1×10^4	19.52	17.38
Interassay	1×10^7	20.22	13.37
	1×10^5	21.81	12.28

[a] NT, not tested.

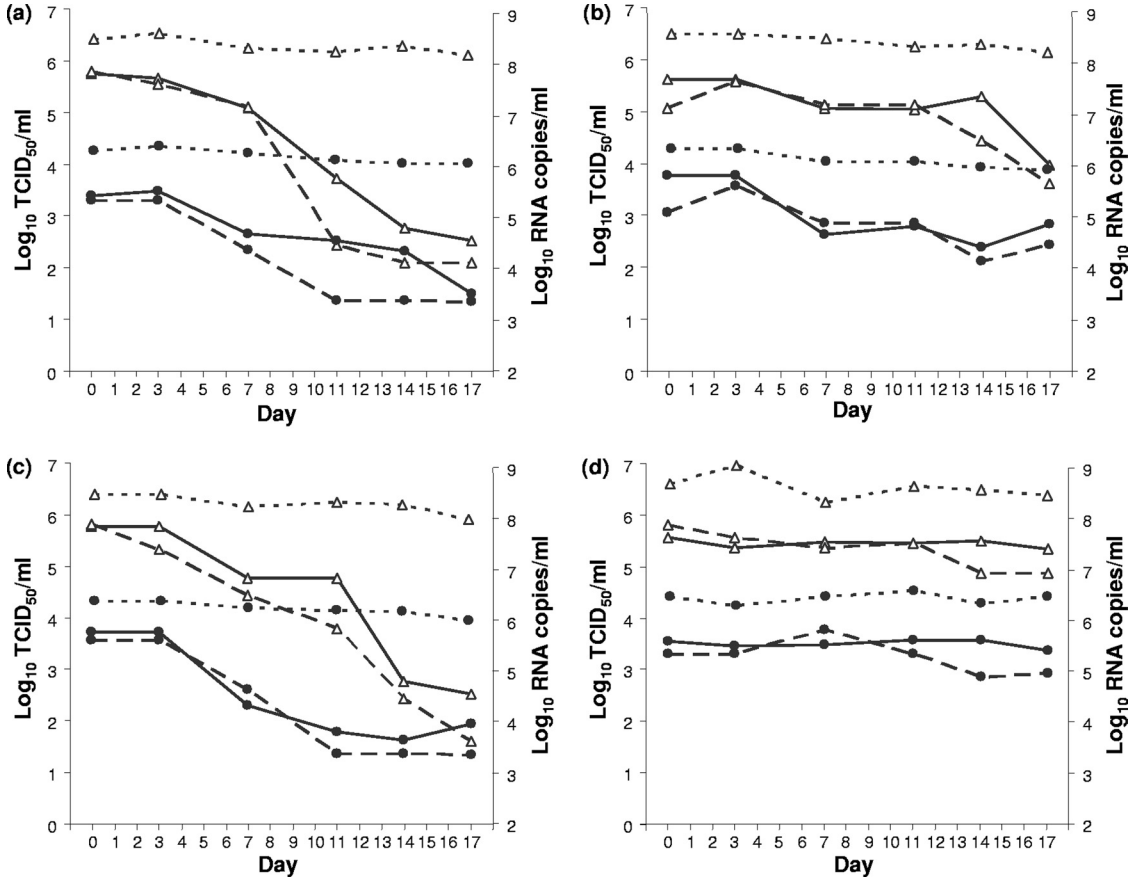

FIG. 4. Monitoring of H5N1 persistence in artificially inoculated water samples over a 17-day period at 4°C. (a) River water (salinity, 0.95 ppt; pH 7.95); (b) lagoon water (salinity, 43.04 ppt; pH 8.53); (c) brackish water (salinity, 6.19 ppt; pH 8.72); (d) double distilled water. The open triangles and filled circles indicate water with initial H5N1 concentrations of $10^{5.7}$ and $10^{3.7}$ $TCID_{50}$/ml, respectively. The dashed lines indicate log virus titers determined using chicken embryo fibroblasts, the solid lines indicate log virus titers determined by qRT-PCR using virions adsorbed to CRBCs, and the dotted lines indicate total log numbers of HA copies detected by qRT-PCR without CRBCs.

ples tested. The standard deviation of the TMV C_T values in each real-time PCR run ranged from 0.17 to 0.35 for the samples extracted without CRBCs and from 0.18 to 0.36 for the samples containing CRBCs. A mean delay of 0.36 cycle for TMV was observed for the samples that contained the recommended amount of CRBCs compared to the samples without CRBCs. Student's *t* test was used for statistical comparison of the corresponding C_T values from each real-time PCR run, and the results showed that these values were not significantly different for the two sample groups ($P > 0.05$). In an experiment in which additional samples were extracted using 4-fold more CRBCs (0.8 ml from a 10% suspension), a mean delay of 1.78 cycles for the TMV internal control was observed compared to the samples containing the recommended amount of CRBCs. In this case a significant difference ($P = 0.001$) was found in the C_T values for the two sample groups, which indicated the presence of RT-PCR inhibitors due to the larger amount of CRBCs. Approximately 4% of the samples tested using the recommended amount of CRBCs exhibited a delay in the TMV C_T, indicating possible inhibition of the RT-PCR. These AIV quantification results were not included in the analysis, and extraction and quantification of the sample were repeated in a subsequent run.

DISCUSSION

Understanding the epidemiology of H5N1 virus is important for the prevention of human, wildlife, and domestic animal disease caused by this virus. Environmental persistence or inactivation of the virus in the various aquatic habitats (lakes, rivers, ponds) of wild bird populations may play an important role in its transmission within and among populations and its maintenance in the environment over time.

A simple technique for detection and isolation of H5N1 AIV from large volumes of natural water was described recently (15). In that study, particles of a nonpathogenic reverse-genetic virus carrying the HA and neuraminidase (NA) genes of an H5N1 virus were isolated from water using fixed CRBCs and ECEs. In the present study, we evaluated the use of H5N1 HPAIV particles adsorbed to CRBCs for quantifying infectious viruses in environmental water using real-time RT-PCR.

Environmental water sampling may be valuable for assessment of the presence, the diversity, and the dissemination of influenza viruses in defined geographical locations since it can complement bird sampling, in comparison to which it is much easier and faster (16).

RT-PCR is a culture-independent method that has been

used for detection of influenza viruses in environmental reservoirs (14, 16, 40). However, the presence of infectious particles was not proven in most cases because no isolation of infectious virus in ECEs was performed. An assumption was made that amplification of influenza virus sequences resulted from RNA extracted from intact virus particles because it was assumed that naked RNA genomes are usually degraded rapidly in environmental water (16, 35). Although real-time PCR results have been correlated with infectious doses of H5 and H7 AIVs from samples obtained from clinically affected birds (17), the different rates of virus inactivation under various aquatic environmental conditions prohibit correlation of the number of detected RNA copies with the infectious virus titer for each sample. This was demonstrated in our experiment using environmental water artificially inoculated with H5N1 virus. No correlation of the number of detected RNA copies with the virus titer over the 17-day incubation period was apparent when RNA was directly extracted from the water (Fig. 4). However, this was not the case when CRBCs were used. We assume that virus particles adsorbed to CRBCs are intact and therefore that the virus titer can be correlated with infectivity. In the case of direct extraction of RNA from water, particles that are defective due to degradation of the viral envelope are expected to appear over time. Although such particles are not infectious, their RNA is still protected from degradation in the matrix protein and the core, and consequently it is detected by qRT-PCR.

At high ambient temperatures, NA promotes release of influenza virus from cells. In our experiment we used zanamivir as an NA inhibitor to avoid release of AIV from CRBCs during the virus adsorption stage. All H5N1 adsorption experiments in this work were conducted at room temperature using zanamivir. However, no difference in recovery of H5N1 was found with incubation at 4°C when zanamivir was omitted (data not shown).

Our results show that real-time PCR using fixed CRBCs is a feasible alternative to traditional methods for detection of virus in water samples for several reasons. Conventionally, isolation of AIV from water is performed by cultivating samples in ECE, which is labor-intensive and takes at least 7 days. A large number of ECEs is required to titrate virus. This system uses ECE as a growth medium, and it is expected that adaptation of individual viral strains to eggs and the possible presence of bacterial or virus contamination or inhibitors from the water can result in a failure to detect or in misinterpretation of the actual infectious virus titer *in vivo*. Moreover, increased biosecurity facilities are needed if the presence of H5N1 HPAIV isolates is examined since replication of virus in ECEs is involved.

Compared to the traditional ECE isolation methods, the method described here is rapid, reduces the handling of infectious material, is not vulnerable to inhibition, can directly identify and quantify infectious H5 AIV, and is expected to discriminate the H5 viral subtype when there are mixed AIV infections. Real-time PCR using fixed CRBCs is a sensitive method. In the present study, it was shown that this method was able to reproducibly detect H5N1 HPAIV in water at a concentration as low as 0.91 EID_{50}/ml or 0.057 $TCID_{50}$/ml. When the titration assays with CEFs and ECEs are used directly with undiluted water samples, they have minimal detection limits (15.85 $TCID_{50}$/ml and 3.98 EID_{50}/ml, respectively), and therefore, the qRT-PCR using AIV particles adsorbed to CRBCs described here is 280 times more sensitive than the former assay and 4.37-fold more sensitive than the latter assay. Most importantly, the number of RNA copies detected using H5N1 HPAIV particles adsorbed to CRBCs correlates with the virus $TCID_{50}$ titer determined in three different (in terms of physicochemical properties and impact on the virus inactivation rate) types of environmental water, which may be indicative of the possibility of infectivity and virus transmission. It was estimated that there were approximately 17.4 HA RNA copies per infectious virus particle, and this presumably reflected the ratio of defective viral genomes to infectious viral genomes in the mature particles.

Good reproducibility is a requirement for quantitative assays, and the very low inter- and intra-assay variability of the qRT-PCR results (Table 3) is comparable to the variability of the results with other protocols (7, 9). This assay's sensitivity or linearity at low concentrations of target HA nucleic acid was not impaired by the simultaneous detection of the TMV internal control, indicating that the reaction conditions ensure that the competition for the reagents in this qRT-PCR does not affect its accuracy. This was accomplished by using high concentrations of *Taq* DNA polymerase, dNTPs, and Mg^{2+}, as proposed previously for multiplex RT-PCR formats (6, 21). A two-step qRT-PCR protocol (i.e., a reverse transcription reaction using random hexamers, followed by a real-time PCR detection) was selected as an initial approach for quantification of infectious H5N1 adsorbed to CRBCs and proved to be robust and highly sensitive. The separate RT option allows different real-time PCR protocols (i.e., matrix gene or subtype specific) to be used with the same cDNAs, which can be preserved better than RNA. However, for large-scale application a validated one-step qRT-PCR would be more convenient, especially if fast results for a specific influenza subtype are needed.

Inclusion of an internal control in each sample from the lysis step onward is very important for the accuracy of qRT-PCR results because each sample is monitored for the efficiency of nucleic acid purification, as well as the presence of PCR inhibitors. Several investigators have developed and described qRT-PCR assays using external reference spikes that involve encapsidated RNAs of animal viruses (9, 39) or naked RNAs (7, 23). The use of intact virions rather than purified naked RNA as an internal control is preferable since these virions can also be used to monitor the efficiency of the viral lysis step itself. However, the risk of infection has to be considered when human- or animal-pathogenic viruses are involved, which makes their use for routine analysis problematic in most laboratories.

Increased stability of internal controls is an important physical property that affects the long-term reproducibility of qRT-PCR applications. In the qRT-PCR assay described here whole TMV particles were evaluated for use as an internal control. TMV has an extraordinarily stable virion that has a 85 to 90°C thermal inactivation point and survives for 3,000 days *in vitro* (http://www.ncbi.nlm.nih.gov/ICTVdb), and thus it can be an ideal internal control showing excellent integrity and reproducibility in qRT-PCR applications over long periods of time in a laboratory. Furthermore, TMV is a plant virus and not haz-

ardous to humans or animals, and it is easy to culture and purify large quantities of it.

Real-time PCR has been used for screening of H5 AIVs in field samples and is a reliable alternative to virus isolation in ECEs. More specifically, North American H5 AIVs were successfully detected and quantified from swab samples by targeting 152 bp in the 5′ region of the HA gene (17, 28). The H5 primers and probe targeting the 152-bp region were modified based on Eurasian H5 sequences from isolates obtained from 1995 to 2005, and they were used successfully to detect Eurasian avian lineage H5N1 AIVs (13, 27). These oligonucleotide sequences used in the currently recommended real-time PCR protocols require continual reevaluation as nucleotide changes accumulate over time, particularly in the HA gene, due to evolution of the H5N1 lineage, as described by the World Health Organization/World Organization for Animal Health/Food and Agriculture Organization H5N1 Evolution Working Group (38). In the present study, an influenza virus H5 HA gene-specific PCR primer set and hydrolysis probe were designed using genomic regions conserved in H5N1 AIVs isolated since 2005, and they exhibited high specificity for amplification of subtype H5N1. The targeted genomic region (182 bp) is similar to that identified previously (28), except for the region targeted by primer H5-R1692. However, because of the great sequence variation of the H5 HA genes, the probes and primers were targeted primarily to European/African H5N1 AIVs and Asian H5N1 AIVs. A high number of different available genotypes of H5N1 HA sequences were used for designing these oligonucleotides. *In silico* analysis and examination of 1,495 HA sequences that were available suggested that the genomic regions targeted are suitable for detecting European, African, and Eurasian H5N1 AIVs that circulated after 2005. Nevertheless, slight modification of the primer sequences depending on the evolution and dispersal of diverse H5N1 viruses may be required (e.g., for some isolates belonging to subclade 2.1.3), but we do not recommend any increase in degeneracy since it would probably affect the characteristics of the quantitative PCR.

In conclusion, a qRT-PCR method using CRBCs provides a feasible alternative to isolation of virus in ECEs. The use of erythrocytes facilitates inexpensive and effective concentration of influenza virus particles from large volumes of water. Our results indicate that the method presented has several important advantages, including detection and quantification of infectious H5N1 AIVs with high sensitivity, a wide dynamic range, and reproducibility. In addition, a large number of water samples can be processed, results can be obtained rapidly (6 h), and there is no requirement for BSL-3 facilities since no replication of infectious virus is involved. Finally, inclusion of TMV as an internal control minimizes the risk of generating false-negative and false low-titer results.

We believe that this assay is a good system for rapid high-throughput monitoring of the presence of the H5 HA subtype in environmental water and could be valuable in studies designed to explore the viability and epidemiology of H5N1 AIV in different waterfowl ecosystems. This methodology may also be used to detect other influenza virus subtypes and to assess their presence and distribution in environmental reservoirs. Additional applications could include the fast initial evaluation of decontamination protocols and assessment of health risks. This could have a great impact on decontamination efforts, which is an extremely timely and important topic for public health. However, in all cases, the positive results should be confirmed and validated by methods that include propagation of the virus.

ACKNOWLEDGMENTS

This work was carried out for the INN-FLU project ("Influence of viral proteins of avian influenza virus on the innate immune response of birds"). This project received research funding from the European Community's Sixth Framework Programme (project SSPE-CT-2006-044372).

The work and information presented here reflect only the views of the authors, and the European Community is not liable for any use that may be made of the information contained herein.

We thank Eleni Makrigianni of the Management Body of the Evros Delta National Park for providing the environmental water samples. We also thank P. Loukopoulos and O. Papadopoulos for their constructive comments on the manuscript.

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