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Development of a Real-Time Reverse Transcriptase PCR Assay for Type A Influenza Virus and the Avian H5 and H7 Hemagglutinin Subtypes

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A real-time reverse transcriptase PCR (RRT-PCR) assay based on the avian influenza virus matrix gene was developed for the rapid detection of type A influenza virus. Additionally, H5 and H7 hemagglutinin subtype-specific probe sets were developed based on North American avian influenza virus sequences. The RRT-PCR assay utilizes a one-step RT-PCR protocol and fluorogenic hydrolysis type probes. The matrix gene RRT-PCR assay has a detection limit of 10 fg or approximately 1,000 copies of target RNA and can detect 0.1 50% egg infective dose of virus. The H5- and H7-specific probe sets each have a detection limit of 100 fg of target RNA or approximately 10^3 to 10^4 gene copies. The sensitivity and specificity of the real-time PCR assay were directly compared with those of the current standard for detection of influenza virus: virus isolation (VI) in embryonated chicken eggs and hemagglutinin subtyping by hemagglutination inhibition (HI) assay. The comparison was performed with 1,550 tracheal and cloacal swabs from various avian species and environmental swabs obtained from live-bird markets in New York and New Jersey. Influenza virus-specific RRT-PCR results correlated with VI results for 89% of the samples. The remaining samples were positive with only one detection method. Overall the sensitivity and specificity of the H7- and H5-specific RRT-PCR were similar to those of VI and HI.

Highly pathogenic strains of avian influenza virus (AIV), which are type A influenza viruses, cause severe disease in domestic poultry, including chickens and turkeys. Because of the high morbidity and mortality associated with highly pathogenic AIV, infection of commercial poultry can lead to substantial economic losses as demonstrated by the 1983 to 1984 outbreak in Pennsylvania (3).

Highly pathogenic AIV is considered to be exotic to the United States, although low pathogenic AIV is routinely isolated from turkeys reared in migratory bird flyways in the midwest and from various avian species in live-bird markets in New York and New Jersey (5, 19). The AIVs currently circulating in the live-bird markets of New York and New Jersey have been classified as low pathogenic. However, previous epornitics of highly pathogenic AIV have occurred in regions where low pathogenic AIV had increased in pathogenicity after circulating in the poultry population for a period of time (1, 2, 4, 6, 8, 9, 23). Historically, highly pathogenic AIVs in poultry have only belonged to the H5 and H7 hemagglutinin (HA) subtypes. Therefore, because there is a greater risk for these subtypes to become highly pathogenic, it is important to identify them specifically in surveillance programs.

Currently, virus isolation (VI) in embryonating chicken eggs and subsequent HA and neuraminidase subtyping by serological methods constitute the standard for AIV detection and subtype identification. Although VI in embryonating eggs is a sensitive method, it may take 1 to 2 weeks to obtain results, by which time the results may no longer be relevant. Conversely real-time reverse transcriptase PCR (RRT-PCR) can be a rapid assay; results, including subtyping, may be available in less than 1 day. It can also be less expensive on a cost-per-sample basis than VI in embryonating eggs.

Standard RT-PCR has been previously applied to the detection of avian influenza virus (10, 13, 17, 18) and each of the 15 HA subtypes (10, 13). Additionally, an RRT-PCR assay for influenza virus has been developed; however, it is a two-step RT-PCR, multiplex assay based on human influenza virus sequences for the detection of influenza virus types A and B (22). One-step RRT-PCR with hydrolysis probes, as described by Holland et al. and Livak et al. (7, 11), has been successfully applied to the detection of various RNA viruses (12, 16). RRT-PCR offers the advantages of speed and no post-PCR sample handling, thus reducing the chance for cross-contamination versus standard RT-PCR.

In this study we describe the development of a one-step RRT-PCR assay with hydrolysis type probes for the rapid

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TABLE 1. PCR primer and hydrolysis probe sequences

Specificity	Primer/probe	Sequence[a] (5′–3′)
Influenza A virus	M + 25	AGA TGA GTC TTC TAA CCG AGG TCG
	M − 124	TGC AAA AAC ATC TTC AAG TCT CTG
	M + 64	FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA
Avian H5	H5 + 1456	ACG TAT GAC TAT CCA CAA TAC TCA G
	H5 − 1685	AGA CCA GCT ACC ATG ATT GC
	H5 + 1637	FAM-TCA ACA GTG GCG AGT TCC CTA GCA-TAMRA
Avian H7	H7 + 1244	ATT GGA CAC GAG ACG CAA TG
	H7 − 1342	TTC TGA GTC CGC AAG ATC TAT TG
	H7 + 1281	FAM-TAA TGC TGA GCT GTT GGT GGC A-TAMRA

[a] FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

screening of clinical samples and live-bird markets for type A influenza virus and for the subsequent identification of the H5 and H7 avian influenza virus subtypes.

MATERIALS AND METHODS

RNA extraction. RNA was extracted with the RNeasy kit (Qiagen, Valencia, Calif.) with a modified protocol for fluid samples recommended by the manufacturer. Briefly, 500 μl of swab material from clinical samples was clarified by centrifugation at 12,000 × g for 2 min, or, for previously isolated viruses, 500 μl of chorioallantoic fluid (CAF) was mixed with 500 μl of 70% ethanol and 500 μl of kit-supplied RLT buffer (Qiagen) and the entire sample was applied to the RNeasy spin column. Subsequently the kit protocol for RNA isolation from the cytoplasm of cells was followed. RNA was eluted in 50 μl of nuclease-free water, and 8 μl per RRT-PCR was used for the template.

Hydrolysis probe and primer sets. An influenza virus matrix gene-specific PCR primer set and hydrolysis probe were designed for a region conserved in all type A influenza virus matrix genes (Table 1). In addition, H5- and H7-specific primer sets for conserved regions of the H5 and H7 HA gene sequences were developed. However, because of the large sequence variation of the H5 and H7 genes, the probes and primers were primarily targeted to North American H5 and H7 influenza viruses. All probes were labeled at the 5′ end with the 6-carboxyfluorescein (FAM) reporter dye and at the 3′ end with the 6-carboxytetramethylrhodamine (TAMRA) quencher dye.

RRT-PCR. The Qiagen one-step RT-PCR kit was used with a 20-μl reaction mixture under the following conditions: 0.8 μl of kit-supplied enzyme mixture (including RT and hot-start *Taq* polymerase), 10 pmol of each primer, 0.3 μM probe, 400 μM (each) deoxynucleoside triphosphate, 3.75 mM $MgCl_2$, and 6.5 U of RNase inhibitor (Promega, Madison, Wis.). The RT step conditions for all primer sets were 30 min at 50°C and 15 min at 94°C. A two-step PCR cycling protocol was used for the matrix gene primer set as follows: 45 cycles of 94°C for 0 s and 60°C for 20 s. The H7 PCR cycling conditions were the same as those for the matrix gene except that a 58°C annealing temperature was used for 40 cycles. A three-step cycling protocol was used for the H5-specific PCR as follows: 94°C for 0 s, 57°C for 20 s, and 72°C for 5 s for 40 cycles. All temperature transition rates were set at the maximum transition rate of 20. Fluorescence data were acquired at the end of each annealing step.

RRT-PCR was performed with the ruggedized advanced pathogen identification device (RAPID) thermocycler (a rapid air thermocycler) and software (Idaho Technologies, Salt Lake City, Utah). Positive and negative results of RRT-PCRs were determined by the RAPID autoanalysis software and rechecked manually.

Specificity of primer and probe sets. RRT-PCR with the matrix gene primer and probe set was performed with template RNA from influenza virus isolates representing all HA subtypes (see Table 6) including both avian North American and Eurasian lineage viruses and isolates of human, equine, and swine origin to demonstrate specificity for type A influenza virus. RRT-PCR with the H5 and H7 subtype-specific probe sets was also performed with template RNA from all HA subtypes listed in Table 6 to demonstrate specificity for their respective subtypes.

In vitro transcription. In vitro-transcribed matrix, H5, or H7 gene RNA was used for positive controls and for the determination of the detection limits of the assay. The influenza H5 and H7 HA genes and the matrix gene which had been cloned into the pAMP1 vector (Life Technologies, Rockville, Md.) as previously described (20) were transcribed with the RiboMax (Promega) kit from the T7 promoter in accordance with the kit instructions and quantitated by spectrophotometer.

Sequencing. The entire matrix gene from six samples which were negative by the RRT-PCR assay and positive by VI and another six samples which were positive by RRT-PCR and VI during the original testing were amplified by standard RT-PCR with the Qiagen one-step kit, and primers were directed to the 12 or 13 conserved bases at the ends of each influenza virus RNA segment. The amplicons were subsequently excised from the gel and extracted with the Qiagen gel extraction kit. If no amplicon was visible, bands at the appropriate size on the gel were excised and extracted from the gel. Products of the gel extraction were cloned into the pAMP1 vector (Life Technologies) as previously described (20). Sequencing was performed with the ABI BigDye terminator system (Applied Biosystems, Foster City, Calif.).

Embryo titration. Virus was diluted in brain heart infusion broth with 10,000 IU of penicillin G, 2,000 μg of streptomycin, 1,000 μg of gentamicin, 650 μg of kanamycin, and 20 μg of amphotericin B/ml. Fertile chicken eggs were inoculated with 100 μl of virus at 10 days of incubation by the chorioallantoic sac route. Five eggs were inoculated per dilution. At 7 days postinoculation CAF was collected and tested for hemagglutination with 0.5% chicken red blood cells (CRBCs) in phosphate-buffered saline. The virus titer was determined by the Reed-Meunch method (15).

RNA for RRT-PCR was extracted from the virus dilutions at the time of egg inoculation with the RNeasy kit (Qiagen) as previously described.

Comparison with VI in embryonating chicken eggs. Fifteen hundred fifty swab samples were obtained from the live-bird markets of New York and New Jersey. Each sample contained a pool of up to five cloacal, tracheal, or environmental swabs collected in 2 ml of brain heart infusion broth. Tracheal and cloacal swabs were obtained from up to five birds of each lot (different birds of the same species and from the same source which entered the market at the same time) present at the time the market was sampled, except waterfowl, from which only cloacal swabs were obtained. Five environmental swabs were taken in the following areas in each market: the office, bird area, slaughter area, and red meat area if present.

RNA was extracted from each sample as previously described. Each sample was tested with the influenza virus matrix primer set. Samples positive with the matrix primer set were subsequently tested with the H7-specific primer set. Samples that were positive with the matrix gene primers and negative with the H7 primer set were then tested with the H5 primer set.

Isolation of influenza virus from swab samples was performed in embryonated chicken eggs. Antibiotics and antimycotics were added to each sample to the following final concentrations: penicillin G, 10,000 IU/ml; streptomycin, 2,000 μg/ml; gentamicin, 1,000 μg/ml; kanamycin, 650 μg/ml; amphotericin B, 20 μg/ml. Four 9- to11-day-old chicken embryos were each inoculated with 300 μl of sample with antimicrobials by the chorioallantoic sac route. The eggs were incubated for 4 days and candled daily for viability; embryos that died within 24 h of inoculation were discarded as nonspecific. CAF from dead and surviving embryos was tested for hemagglutination of 0.5% CRBCs in phosphate-buffered saline. Samples from dead embryos that were negative for hemagglutination were passaged a second time.

HA subtypes of all hemagglutination-positive samples were determined by hemagglutination inhibition (HI) assay (18). Hemagglutinating CAF was standardized to 4 HA units, and HA was mixed with an equal volume of influenza virus subtype reference serum at a titer between 1:32 and 1:64. Reference serum and CAF were incubated for 30 min at room temperature, and 0.5% CRBCs

TABLE 2. Summary of RRT-PCR and VI results for individual samples from live-bird markets tested for type A influenza virus

Result by:		No. of samples (total, 1,550)
RRT-PCR	VI	
Pos.	Pos.	202
Neg.	Neg.	1,183
Pos.	Neg.	64
Neg.	Pos.	101

TABLE 4. Summary of RRT-PCR and HI results for individual samples from live-bird markets tested for the H7 or H5 HA subtype

Result by:		No. of samples	
RRT-PCR	HI	H7 (total, 202)	H5 (total, 8)
Pos.	Pos.	194	1
Neg.	Neg.	4	7
Pos.	Neg.	1	0
Neg.	Pos.	3	0

were added and mixed. The assay was evaluated for HI after incubation at room temperature for 30 min.

Results of the RRT-PCR assay were compiled with the results of VI and HI by an independent third party to prevent bias.

RESULTS

Comparison of RRT-PCR with VI and HI. The sensitivity and specificity of the RRT-PCR assay were compared to those of VI in embryonating eggs with 1,550 clinical swab samples from New York and New Jersey live-bird markets. Of the swab samples tested by both RRT-PCR and VI, 1,183 were negative by both assays and 202 were positive by both assays (Table 2). Overall the results of the two assays agreed on 1,385 samples (89%) and disagreed on 165 samples (11%). Of the 165 samples on which results for the assays differed, 101 samples were positive by VI and negative by RRT-PCR; RRT-PCR detected 202, or 66.6%, of the 303 samples that were positive by VI. Sixty-four samples were positive by RRT-PCR but negative by VI; VI detected 75.6% of the samples which were RRT-PCR positive.

RRT-PCR and VI were also compared for their relative sensitivities and specificities for the detection of positive live-bird markets. A market was considered positive if at least one sample was positive. Of the 109 live-bird markets tested in the study, 37 were negative by both assays and 61 were positive by both assays. Overall the assays agreed on 98 markets (90%) (Table 3). Among the markets with differing results, four were positive by VI and negative by RRT-PCR and seven markets were positive by RRT-PCR but negative by VI. There was at least one RRT-PCR-positive sample in 61, or 93.8%, of the 65 markets that were positive by VI.

RRT-PCR for H5 and H7 was compared to HI for sensitivity and specificity. Two hundred two samples were tested for H7 by both HI and RRT-PCR. There were four samples negative by both assays, and 194 samples were positive by both assays; results for 98% of the samples were in agreement (Table 4). One sample was positive by RRT-PCR and negative by HI; three samples were positive by HI but negative by RRT-PCR. Because H5 RRT-PCR was performed only on samples that were influenza virus positive and H7 negative, only eight samples were tested by both methods. Both tests correlated on all samples tested for H5; one sample was positive, and seven samples were negative (Table 4).

The ability of the RRT-PCR assay to detect H5 and H7 was also determined for individual live-bird markets. Both HI and RRT-PCR were used to determine the subgroups of AIV present in 60 markets; 56 of the markets were positive by both methods for the presence of H7 subtype virus (Table 5), and two markets were negative by both methods (overall 96.6% correlation). The remaining two markets were positive for H7 by HI and negative for H7 by RRT-PCR. Four markets were tested with both methods for H5. Both assays correlated for all four markets; one was positive, and three were negative.

Assay sensitivity and specificity. The sensitivity of the influenza virus matrix gene, H5, and H7 RRT-PCR assays relative to virus titer detectable by standard VI in embryonating eggs was determined. The detection limit of the matrix gene assay was determined to be approximately 10^{-1} 50% egg infective dose (EID_{50}) reproducibly by using the RNA extraction protocol described in Materials and Methods. The assay could detect RNA from 10^1 EID_{50}s of H5 or H7 AIV.

Detection limits for AIV- and the HA subtype-specific probe sets were determined by detection of in vitro-transcribed matrix gene, H5 HA gene, or H7 HA gene RNA. The minimum copy number of matrix gene RNA which could be detected was approximately 10^3 gene copies or 10 fg of in vitro-transcribed RNA. Both the H5 and H7 primer sets had a reproducible detection limit of 10^3 to 10^4 gene copies or approximately 100 fg of in vitro-transcribed RNA.

The matrix gene primer and probe set was tested with RNA obtained from avian-origin influenza virus isolates representing all 15 HA subtypes and isolates of human, equine, and swine origin. The matrix primer set was able to detect all type A influenza viruses tested including viruses of human, equine, and swine origin (Table 6). The H5 and H7 primer sets were

TABLE 3. Summary of RRT-PCR and VI results for live-bird markets tested for type A influenza virus

Result by:		No. of markets (total, 109)
RRT-PCR	VI	
Pos.	Pos.	61
Neg.	Neg.	37
Pos.	Neg.	7
Neg.	Pos.	4

TABLE 5. Summary of RRT-PCR and HI results for live-bird markets tested for type A influenza virus and the H7 or H5 HA subtype

Result by:		No. of markets	
RRT-PCR	HI	H7 (total, 60)	H5 (total, 4)
Pos.	Pos.	56	1
Neg.	Neg.	2	3
Pos.	Neg.	0	0
Neg.	Pos.	2	0

TABLE 6. Results of RRT-PCR with type A influenza virus-, H5-, and H7-specific primer and probe sets by type A influenza virus isolate and subtype

Isolate	Subtype	Test specificity for:		
		Type A influenza virus	H5	H7
Duck/NJ/7717-70/95	H1N1	+	−	−
Mallard/NY/6750/78	H2N2	+	−	−
Env/NY/19019-6-98	H3N8	+	−	−
Duck/Victoria/9211-18-1400/92	H3N8	+	−	−
Duck/Alberta/286/78	H4N8	+	−	−
Chicken/Puebla/8629-602/94	H5N2	+	+	−
Chicken/MA/11801/86	H5N2	+	+	−
Avian/NY/31588-2/00	H5N2	+	+	−
Chicken/NJ/17169/93	H5N2	+	+	−
Mallard/OH/184/86	H5N1	+	+	−
Duck/Malaysia/97	H5N3	+	+	−
Chicken/NY/14677-13/98	H6N2	+	−	−
Turkey/PA/7975/97	H7N2	+	−	+
Chicken/PA/13552-1/98	H7N2	+	−	+
Quail/AR/16309/94	H7N3	+	−	+
Chicken/NY/8030-2/96	H7N2	+	−	+
Chicken/NewSouthWales/1688/97	H7N4	+	−	+
Turkey/Ontario/6118/67	H8N4	+	−	−
Chicken/NJ/1220/97	H9N2	+	−	−
Chicken/Korea/96006/96	H9N2	+	−	−
Chicken/Germany/N/49	H10N7	+	−	−
Turkey/VA/31409/91	H10N7	+	−	−
Chicken/NJ/15906-6/96	H11N1	+	−	−
Duck/England/56	H11N1	+	−	−
Duck/LA/188B/87	H12N5	+	−	−
Gull/MD/704/77	H13N6	+	−	−
Mallard/Gurjev/263/82	H14N5	+	−	−
Shearwater/W.Australia/2576/79	H15N6	+	−	−
Aichi/68	H3N2	+	−	−
Equine/KY/211/87	H3N8	+	−	−
Swine/MN/9088/99	H3N2	+	−	−
Swine/IN/1726/89	H1N1	+	−	−

tested with avian-origin isolates representing viruses of each HA subtype (Table 6). The H5 and H7 primer and probe sets detected RNA only from virus isolates of their respective subtypes.

Sequencing. Sequencing the matrix genes of six individual samples that were VI positive from the live-bird market clinical samples but RRT-PCR negative revealed no sequence variation in the regions where the M+64 probe is located. One isolate had a single base change of an A to a G, which was located at the 5′ base of the M+25 primer, and there was also a single base change of a T to a C at position 7 from the 5′ end of the M−124 primer in all isolates sequenced including the six isolates which were positive by RRT-PCR during the original testing.

DISCUSSION

Because mildly pathogenic AIV infection does not cause any pathognomonic clinical signs and is commonly subclinical, the presence of the virus must be determined by a diagnostic test. We have developed RRT-PCR as a rapid alternative to VI for AIV detection and subtyping. In addition to its speed, RRT-PCR reduces the handling of infectious material compared to VI. RRT-PCR also offers several advantages over standard RT-PCR such as speed and elimination of the possibility of cross-contamination of new samples with previously amplified products because the sample tube is never opened after PCR. Additionally, because the RRT-PCR product is detected with a sequence-specific probe, there is confirmation that the correct target was amplified, reducing the chances for false positives.

The RRT-PCR assay for AIV was developed to be a rapid screening tool for commercial flocks and live-bird markets. Importantly, in this study, results could be obtained quickly; 28 clinical samples could be processed and tested in approximately 3 h. The assay also performed well compared to VI and HI since 94% of the VI-positive markets were also positive by RRT-PCR and 97% of the markets that were H7 positive by HI were H7 positive by RRT-PCR. However, RRT-PCR did not correlate as well with VI by individual sample tube, as 11% of the samples were positive by only one method.

Differences in the detection of AIV between the assays can probably be explained at least in part by what the assays are detecting. VI can detect only live virus, and virus that has been inactivated during shipping or by disinfectants (which may be present in environmental samples) will not be detected, whereas they may potentially be detected by RRT-PCR. Additionally, all influenza viruses may not readily adapt to growth to detectable titers in embryonating chicken eggs within two passages. This may explain why some samples were RRT-PCR positive and VI negative. Interestingly, 26 of 64 (40.6%) RRT-PCR-positive, VI-negative samples were classified as non-H7 and non-H5 subtype isolates.

Factors that may adversely affect the sensitivity of the RRT-PCR assay versus VI include RT-PCR-inhibitory substances in the samples, the use of a one-step RT-PCR method, inefficient RNA extraction procedures, and the potential for RNA to rapidly degrade before testing. Importantly, this RRT-PCR test was designed to balance both sensitivity and ease of use so that the procedure could be performed rapidly and on a large scale. Therefore, a single-step RT-PCR was used, although this method is reported to be less sensitive than a two-step RT-PCR procedure (14). A single-step RT-PCR procedure also greatly reduces the risk of cross contamination because the tubes are not reopened once the template is added. Additionally, VI tests use an equivalent of nine times more volume of the original sample than is used for RRT-PCR; therefore it is possible that alternative RNA extraction methods utilizing a greater sample volume would increase the sensitivity for samples with a low virus titer. Finally, RNA is relatively unstable and RNA-degrading enzymes are ubiquitous; therefore the RNA may have been degraded prior to testing, although efforts were made to reduce this possibility.

Sequence variation in the matrix gene may also explain why the RRT-PCR test failed to detect viral RNA in some of the virus-positive samples. However matrix gene sequences from several samples that were positive by VI but negative by RRT-PCR did not have any sequence variation in the probe binding region, and the single base change present in the M−124 primer is present in many other isolates that were detected with these primers, including six RRT-PCR-positive samples from the same study. The 5′ base change in one isolate in the M+25 primer binding region is also probably not adequate to prevent primer binding. In general, the region of the matrix

gene to which the primers and probe are directed is conserved among type A influenza viruses; therefore this assay should detect most, if not all, type A influenza viruses, as demonstrated by the detection of nonavian isolates with this primer and probe set.

Correlation between the assays was better with H7 and H5, although few samples were tested for H5 and H7, and only samples that were positive for influenza virus by both tests can be compared. For example, it is not known if a sample which was positive by VI and H7 HI would have been positive by the H7 RRT-PCR, although it was negative by the influenza virus matrix gene RRT-PCR test. Although the H5 test detected all of the North American avian-lineage H5 isolates tested, too few clinical samples were tested to definitively validate the H5 RRT-PCR test versus HI.

A comparison of EID_{50} with RRT-PCR indicated that the matrix gene primer and probe set was able to detect 10^{-1} EID_{50} of virus. The increased sensitivity of the matrix gene RRT-PCR versus EID_{50} may be due to the ability of the RRT-PCR assay to detect fewer virions than the minimum necessary to cause hemagglutination. RRT-PCR is also expected to detect noninfectious particles, which have been estimated to constitute as much as 90% of some virus preparations (6). It is unclear why increased sensitivity relative to EID_{50} was not observed with the H5- and H7-specific assays.

We have developed an RRT-PCR assay for the detection of type A influenza virus and the avian H5 and H7 HA subtypes. RRT-PCR is less expensive than VI, and, importantly, results are available much faster. This assay provides a rapid and feasible alternative to VI in embryonating chicken eggs and subtyping by HI as a flock or live-bird market screening tool.

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