Direct Sequence Detection of Structured H5 Influenza Viral RNA

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We describe the development of sequence-specific molecular beacons (dual-labeled DNA probes) for identification of the H5 influenza subtype, cleavage motif, and receptor specificity when hybridized directly with *in vitro* transcribed viral RNA (vRNA). The cloned hemagglutinin segment from a highly pathogenic H5N1 strain, A/Hanoi/30408/2005(H5N1), isolated from humans was used as template for *in vitro* transcription of sense-strand vRNA. The hybridization behavior of vRNA and a conserved subtype probe was characterized experimentally by varying conditions of time, temperature, and Mg²⁺ to optimize detection. Comparison of the hybridization rates of probe to DNA and RNA targets indicates that conformational switching of influenza RNA structure is a rate-limiting step and that the secondary structure of vRNA dominates the binding kinetics. The sensitivity and specificity of probe recognition of other H5 strains was calculated from sequence matches to the National Center for Biotechnology Information influenza database. The hybridization specificity of the subtype probes was experimentally verified with point mutations within the probe loop at five locations corresponding to the other human H5 strains. The abundance frequencies of the hemagglutinin cleavage motif and sialic acid recognition sequences were experimentally tested for H5 in all host viral species. Although the detection assay must be coupled with isothermal amplification on the chip, the new probes form the basis of a portable point-of-care diagnostic device for influenza subtyping. (J Mol Diagn 2008, 10:225–235; DOI: 10.2353/jmoldx.2008.070094)

the most devastating, that of 1918, caused at least 50 million deaths worldwide.¹ It is widely believed that a future influenza pandemic is inevitable and that such an event could result in global mortality in the range of tens to hundreds of millions if unchecked.²

Structural genes coding for two viral proteins, hemagglutinin (HA) and neuraminidase (NA), specifically identify influenza subtype. Influenza viruses have the propensity to undergo genetic reassortment resulting in novel subtypes that present altered antigenic surfaces for interaction with immune cells and are therefore not recognized by pre-existing antibodies.³ This phenomenon, antigenic shift, may presage a pandemic. Sixteen HA subtypes and nine NA subtypes have been identified to date. Although most have not been associated with human disease, a future pandemic may result from one of the many possible permutations of these types.⁴

The potential health threat posed by various influenza subtypes is dependent on the intrinsic virulence of the virus, the transmissibility in humans, and the level of type-specific immunity within a population. The specter of a pandemic has recently been raised by the occurrence of severe human disease due to subtype H5N1, a novel form of avian influenza, in Asia.⁵ Despite a relatively low incidence of human disease from influenza A H5N1 thus far, the number of cases will rise exponentially should the virus adapt and result in sustained and efficient human-to-human transmission.⁶ The ability to rapidly identify influenza subtypes is essential to provide key information in real-time fashion to health care workers and public health professionals, and to facilitate the expeditious implementation of responses such as community mitigation strategies and antiviral and vaccine deployments in emergent infections (World Health Organization Guidelines for the Collection of Human Specimens for Laboratory Diagnosis of Avian Influenza Infection, http://www.who.int/csr/disease/avian_influenza/guidelines/humanspecimens/en/print.html; accessed July 2007). An ideal subtype-specific identification method would incorporate the specificity of genetics-based analyses,^{7–9} the information content of an array-based or sequencing method,^{10–13} and the speed and simplicity of a one-step test.^{14–19} In this work, we describe a solution phase, sequence-specific hybridization methodology using dual-labeled probes

Influenza continues to threaten human health both in its seasonal form and its potential to cause pandemics. Three pandemics were documented in the 20th century;

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designed to hybridize directly with the viral RNA (vRNA) of interest without the need for thermal cycling.

Dual labeled probes, also known as molecular beacons, are stem loop structures of single-stranded DNA, where one end is labeled with a fluorophore and the other end a quencher molecule.^{20–23} In the absence of a target sequence, the complementary ends of the probe form a 5- to 7-bp stem by hybridization, which quenches the fluorescent signal. The fluorophore unquenches and fluoresces when the 15- to 25-base loop hybridizes to the complementary RNA target sequence with higher affinity than the stem. Availability of a probe binding site on the RNA depends on conformation, which may change either spontaneously or induced through binding of probes.

Native RNA structure, which was investigated using molecular beacons on ribosomes,^{21,24} may be determined by folding kinetics rather than by thermodynamics. Thermodynamically, the values for free energies for formation of nearest neighbor base pair from single strands and for the formation of secondary structures can be altered by changing temperatures. Prediction of secondary structure *in silico* is limited by the accuracy with which the empirical parameters can be determined and by principal deficiencies, for example by the lack of energy contributions resulting from tertiary interactions.^{24–26} Therefore, experimental measurement of a probe response to RNA best confirms their activity.²⁷ Detection of potato leaf roll virus RNA was combined in a molecular beacon assay with a continuous T7 polymerase amplification providing “real-time” detection.²⁸ Experimental parameters include temperature, concentration and cations. Tsourkas et al studied the thermodynamics of RNA hybridization to molecular beacons with and without protective modifications.²⁹ However, the critical role of divalent cations in disruption or stabilization of influenza RNA structures is not known.^{30,31} RNA phosphate groups are believed to provide strong binding sites for Mg^{2+} , making secondary or tertiary structures particularly sensitive to concentration.

Although molecular beacons have been used in polymerase chain reaction assays, for parainfluenza detection,³² and in live cell detection of RSV virus,³³ this is the first report to our knowledge detailing the use of molecular beacons for direct detection and subtyping of influenza viral RNA. This work elucidates the design of high information content probes for the HA segment of the H5 influenza genome, experimentally characterizes the interaction of a molecular beacon with H5 viral RNA, and demonstrates the detection method given the proper conditions of vRNA concentration and probe. In particular, the work highlights the effects of secondary structure of full-length RNA on the hybridization and identification of pathogenic strains by sequence. These probes are intended for use with our continuous flow microfluidic reactor,³⁴ modified to isothermally amplify full-length sense-strand RNA identical in sequence to vRNA. These sequences are then distributed to separate wells containing one of the beacons described herein.

Materials and Methods

In Vitro Synthesis of Nonpathogenic Viral RNA

A wild-type influenza A H5 DNA sequence from the National Center for Biotechnology Information (NCBI), accession number AB239125 [A/Hanoi/30408/2005(H5N1)], coding for the hemagglutinin segment was synthesized (DNA 2.0, Menlo Park, CA), inserted into a pJ10 *Escherichia coli* propagation plasmid, and sequenced. A truncated SP6 RNA polymerase promoter, which lacks a G at position 1, is followed by the reverse complement of this H5 cDNA sequence. The 3' end of the sequence includes a restriction site for *Kpn*2I, which cleaves the sequence TCCGGA one base in from the 5' ends on both strands. Plasmid cleavage with this enzyme produces a 3' overhang that is removed using the Klenow fragment of DNA polymerase I. The SP6 transcription produces H5 viral RNA with native 5' and 3' ends. The restriction digest was separated on an Agilent 7500 DNA chip. Transcription products were visualized on an Agilent 6000 Nano RNA microchip. The *in vitro* transcribed RNA was reverse transcribed to cDNA, inserted into a plasmid and sequenced to confirm the product was correct. These plasmid-derived DNA sequences were used for generating non-pathogenic viral RNA and referred to in this paper as H5 vRNA.

Molecular Beacon Design

To locate suitable 16- to 25-nucleotide conserved regions for designing subtype probes, cDNA sequences from seasonal human influenza subtypes were downloaded from the NCBI Influenza Virus Resource. Within a given subtype, sequences were aligned to locate conserved regions of the genome across the species for the subtype. AlleleID 4.0 (Premier Biosoft International, Palo Alto, CA) searched for probe candidates with optimized thermodynamic parameters of hybridization by screening the full length of the cDNA. Probes for motif identification were designed by downloading accession numbers for all H5, either for humans or all hosts, for use as a master key. Potential motif targeting nucleotide sequences were used as search strings in the NCBI database and matches were downloaded. Sequences not detected were discovered by comparison to the master key accession list. The non-detected (mutated) nucleotide sequences were scanned to locate the target protein motif based on open reading frame information contained in the NCBI records. Point mutation probes were designed and iteratively used as search strings until all nucleotide sequences for the target motif were discovered. Records containing partial sequence data that did not contain the motif of interest were not included in the statistics. Additional nucleotides, spanning regions beyond the target motif, were recorded to ensure the probe target sequence retained a T_m greater than the stem. The probe sequences were designed to match the NCBI record of cDNA, which is complementary to the viral RNA. Complementary DNA sequences and probes were synthesized commercially (IDT DNA, Coralville, IA) to specification.

Table 1. Frequency of Target Sequences in the H5N1 Hemagglutinin Population

Probe ID	Human hosts		All hosts		Probe sequence*
	True+	% population	True+	% population	
H5G1192	145	100.0	954	61.4	5'- CGCGA AGGCAATAGATGGAGTCACT CGCG -3'
H5G1112	125	86.2	991	63.8	5'- CGCGA ATGGTAGATGGTTGGTATGGGTAT CGCG -3'
H5R734	87	60.0	913	58.8	5'- CGCTA ACGGGCAAAGTGAAGGAG CG -3'
H5C1046	71	49.0	638	42.9	5'- CGATG AGAGMAGAAGAAAAAGAGAG GCATCG -3'
H5C1045	1	0.7	32	2.1	5'- CGATC AAAGAGAGAGAAGAAAAAGAGAT CG -3'
All H5	145	100.0	1554	100.0	N/A

*Sequences in boldface are in the stem structure and do not bind the target region.

Fluorescence Measurements

The time response of the IowaBlack-quenched, 5-carboxyfluorescein-labeled influenza probes to cDNA and vRNA was measured (ex/em 485/515) on a QM-4/2005SE spectrophotometer (Photon Technologies International, Birmingham, NJ). Sample exposure to light was reduced using a shutter, which cycled every 5 seconds. Data were collected at 2 Hz. A temperature-controlled Peltier jacket heated and cooled the 10-mm path length, 50- μ l quartz cuvette (Starna Cells, Atascadero, CA) at 1.5°C/second and 0.5°C/second, respectively. The fluorescence of each molecular beacon was evaluated over time after addition of its complementary DNA or RNA target sequence. A shutter control system was used to limit photobleaching. All probes in 20 mmol/L Tris, pH 8, were first evaluated at 30 nmol/L using excess short single-strand DNA fragments complementary to the probe loop at 45 nmol/L. Viral RNA measurements were performed using RNase-free reagents and cuvettes decontaminated with RNaseZAP (Ambion, Austin, TX), thoroughly rinsed, and dried. In all experiments, the cuvette was blanked with buffer and background fluorescence intensity of probes in the absence of target was recorded before the addition of target sequences. The fluorescence reading was paused, the 55- μ l cuvette contents withdrawn, mixed into 10 μ l of DNA target, and then rapidly returned to the cuvette for fluorescent measurement over time.

Steady-State Fluorescence and Hybridization Kinetics

The kinetic constants for probe hybridization to DNA and RNA targets were calculated by fitting the fluorescence response curve, $F(t)$, to the first-order rate equation, $F = A(1 - e^{-t/\tau})$, where A is the steady-state fluorescence and τ is the hybridization time. The steady-state fluorescence time and the rate of hybridization, τ^{-1} , can be derived from this calculation. Note that alternate rate equations can also be used for describing the hybridization kinetics.

Results

Influenza Beacon Design

Molecular beacons for H5 RNA were designed *in silico* to target specified nucleotides in three site-specific regions.

These include a general H5 subtype probe, identifier probes for HA cleavage site, and receptor binding site identifiers shown in Table 1. The probes are numbered based on the first nucleotide of the binding site in our H5 reference sequence, NCBI AB239125 [(A/Hanoi/30408/2005(H5N1)]. Since these probes are not intended for detection with thermocycling processes, the design melting temperature (T_m) is as low as 50°C. The H5G subtype probes targeted more conserved regions^{35,36} of the viral RNA sequences while uniquely identifying the specific viral H5 subtype. Between subtypes, amino acid sequences differ by 20 to 74%, whereas strains within a particular subtype differ by 0 to 9%,³⁷ which enables the design of probes for subtype identification. The second probe group, H5R, targeted coding regions in the hemagglutinin gene for amino acids 226 and 228, which indicate the HA receptor binding preference.³⁸ The binding target, a sialic acid, is specifically attached to host cell surface galactose in either α 2,3-linkages in avian receptors, or α 2,6-sialic acid linkages as found in humans.^{39,40} A third probe group, H5C, evaluated the cleavage motif in the HA protein for the number of consecutive basic amino acids. The hemagglutinin protein is expressed as a single peptide (HA0) and cleaved by host proteases into two subunits, HA1 and HA2. Polybasic sites with five or six basic amino acids, a signature of high pathogenesis, have been present in all outbreaks of the highly pathogenic H5 avian influenza strains (World Health Organization, Avian Influenza ("Bird Flu") Fact Sheet, http://www.who.int/mediacentre/factsheets/avian_influenza/en/index.html; accessed July 2007). Together, the binding preference and the cleavage motif in HA help discriminate between, but not determine, high pathogenic avian influenza and low pathogenic avian influenza,⁴⁰⁻⁴² which is essential to determining the proper health response.

Figure 1 shows the cleavage site amino acid distribution for humans and all hosts. The cleavage sequence for H5 in humans is dominated by two motifs, RRRKKR (58%) and SRRKKR (33%), indicating their high pathogenic nature, whereas the all-host population contains both high pathogenic avian influenza and low pathogenic avian influenza markers, such as PQRETRG, in the "other" category. Table 2 details the sequence specific nucleotide mutations for site cleavage in the H5 strains isolated from humans and all hosts. The cleavage motif probe, H5C1045 (5'-CGCATCAAAGAGAGAGAAGAAA-AAAGAGAATGCG-3'), was designed for the Hanoi strain with five flanking nucleotides on each end that do not

Figure 1. Cleavage motif frequency of the HA segment of H5 influenza isolated from all hosts (A) and humans (B). The cleavage sequence for H5 in humans is dominated by two motifs, RRRKKR (58%) and SRRKKR (33%). The polybasic amino acid sequence of lysine (K) and arginine (R) is a hallmark of high pathogenic H5 strains. The all-host high pathogenic population consists of RRRKKR (46%) and RRRKRG (27%) with only minor contribution from SRRKKR (3%). The sialic acid recognition motif distribution at amino acids 225–230 in H5 HA sequences isolated from all hosts (C) and humans (D). As of January 2007, no H5 sequences in the NCBI database contain the Q226 and G228S mutations, which confer human sialic receptor binding and are present in currently circulating human influenza subtypes.

bind the target sequence. The HA protein is cleaved into two subunits, HA1 and HA2, at a site with basic amino acids that enhance the local charge, whereas the low pathogenic avian strains lack this feature. The sialic acid

receptor preference of HA is primarily determined by the amino acids Leu226 and Ser228 (XLXS) for the human α 2,6 receptor and Gln226, Gly228 (XQXG) for (2,3 avian receptor specificity. Of the human H5 isolates in the NCBI database, 95% (127 of 133) contain the sequence GQSG. As of January 2007, no H5 sequences in the NCBI Influenza Virus Resource contain the Q226 and G228S mutations that would allow for human sialic receptor binding. The probe, H5R734 (5'-CGCGTAACGGG-CAAAGTGGGAAGGAACGCG-3'), was designed for receptor identification of our test strain. Complete descriptions of the sialic acid mutations in the H5 population are listed in Table 3. Since the number of nucleotide sequences is not equally represented by protein sequences in the NCBI database, small differences exist in the frequency count between Table 1 and Tables 2 and 3.

Population Sensitivity and Specificity

The sensitivity and specificity of the proposed H5 subtype probes to detect H5 variants were screened using the NCBI Influenza Virus Resource (<http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html>; accessed July 2007) due to the limited availability and pathogenicity concerns of handling human H5N1 viral samples. Database screening establishes the theoretical limits of detection. Sensitivity, which is defined as true positives/(true positives + false negatives), quantifies the detection of true H5 sequences offset by H5 mutants the probe does not detect. The specificity, which is defined as true negatives/(true

Table 2. Cleavage Site Protein Motifs and Nucleotide Frequencies for H5N1 Strains

Motif/probe ID	Human hosts		All hosts		Probe binding sequence
	True+	% population	True+	% population	
ERRRKKR	78	54.2	700	47.0	5'-GARAGRAGRMGRAARAARMGRGG-3'
	71	49.3	638	42.9	5'-GAGAGAAGAAGAAAAAGAGAGG-3'
	2	1.4	17	1.1	5'-GAGAGAAGGAGAAAAAGAGAGG-3'
	4	2.8	4	0.3	5'-GAGAGAAGAAGAAAAAGAGGGG-3'
	1	0.7	7	0.5	5'-GAGAGAAGAAGAAAAAGAGG-3'
	0	0.0	3	0.2	5'-GAGAGAAGAAGAAAAAGAGAGG-3'
	0	0.0	2	0.1	5'-GAGAGGAGAAGAAAAAGAGAGG-3'
	0	0.0	2	0.1	5'-GAAAGAAGAAGAAAAAGAGAGG-3'
	0	0.0	11	0.7	5'-GAGAGAAGAAGAAAAAGCAGG-3'
	0	0.0	1	0.1	5'-GAGAGAAGAAGAAAAAGCAGG-3'
	0	0.0	1	0.1	5'-GAAAGAAGAAGAAAAAGCAGG-3'
	0	0.0	8	0.5	5'-GAGAGAAGAAGAAAGAGAGAGG-3'
	0	0.0	5	0.3	5'-GAGAGAAGACGAAAAAGAGAGG-3'
ESRRKKR	56	38.9	57	3.8	5'-GARAGCAGAAGAAAAAGAGAGG-3'
	53	36.8	54	3.6	5'-GAGAGCAGAAGAAAAAGAGAGG-3'
	3	2.1	3	0.2	5'-GAAAGCAGAAGAAAAAGAGAGG-3'
RERRRKR	6	4.2	408	27.4	5'-AGAGARAGAAGAAGAAAAAGAGG-3'
	4	2.8	283	19.0	5'-AGAGAAAGAAGAAGAAAAAGAGG-3'
	0	0.0	1	0.1	5'-AGAGAGAGGAGAAGAAAAAGAGG-3'
	0	0.0	1	0.1	5'-AGAGAGAGAAGAAGAAAGAGAGG-3'
	2	1.4	118	7.9	5'-AGAGAGAGAAGAAGAAAAAGAGG-3'
	0	0.0	3	0.2	5'-AGAGAGAGAAGAAGGAAAAGAGG-3'
	0	0.0	1	0.1	5'-AGTGAAGAAGAAGAAAAAGAGG-3'
	0	0.0	1	0.1	5'-AGAGAAAGGAGAAGAAAAAGAGG-3'
	2	1.4	16	1.1	5'-AGAGAGAAAAGAAGAAAAAGAGA-3'
KRRKKR	1	0.7	32	2.1	5'-AGAGAGAGAAGAAAAAGAGAGG-3'
ERRKKR	1	0.7	1	0.1	5'-AGAGAGAGGAAAAAGAAAAAGAGA-3'
EKRKKR	0	0.0	276	18.5	
Other	144	100.0	1490	100.0	

Table 3. Sialic Acid Receptor Protein Motifs and Nucleotide Frequencies for H5N1 Strains

Probe ID/motif*	Human hosts		All hosts		Probe binding sequence
	True+	% population	True+	% population	
NGQSGR	138	95.8	1475	99.0	5'- AAYGGSCARAGYGGRMGRD -3'
H5R734	87	60.0	909	61.0	5'-AACGGGCAAAGTGGGAAGGA-3'
NGQSGR-A	20	13.9	241	16.2	5'-AACGGGCAAAGTGGGAAGAA-3'
NGQSGR-B	2	1.4	4	0.3	5'-AACGGCCAAAGTGGGAAGAA-3'
NGQSGR-C	16	11.1	60	4.0	5'-AACGGGCAAAGTGGGAGGA-3'
NGQSGR-D	9	6.3	26	1.7	5'-AATGGGCAAAGTGGGAAGGA-3'
NGQSGR-E	2	1.4	2	0.1	5'-AACGGGCAAAGTGGACGGA-3'
NGQSGR-F	2	1.4	8	0.5	5'-AACGGGCAGAGTGGGAGGA-3'
NGQSGR-G	0	0.0	36	2.4	5'-AATGGGCAAAGTGGGAAGAA-3'
NGQSGR-H	0	0.0	47	3.2	5'-AACGGACAAAGTGGGAAGAA-3'
NGQSGR-I	0	0.0	17	1.1	5'-AACGGGCAGAGTGGGAAGAA-3'
NGQSGR-J	0	0.0	5	0.3	5'-AATGGACAAAGTGGGAAGGA-3'
NGQSGR-K	0	0.0	31	2.1	5'-AACGGACAGAGTGGGAAGGA-3'
NGQSGR-L	0	0.0	31	2.1	5'-AATGGACAAAGTGGGAAGAA-3'
NGQSGR-N	0	0.0	39	2.6	5'-AACGGACAAAGTGGGAAGGA-3'
NGQSGR-O	0	0.0	2	0.1	5'-AATGGGCAGAGCGGACGAA-3'
NGQSGR-P	0	0.0	7	0.5	5'-AACGGACAAAGTGGGAGAA-3'
NGQSGR-Q	0	0.0	2	0.1	5'-AATGGACAGAGCGGAAGAG-3'
NGQSGR-R	0	0.0	1	0.1	5'-AATGGGCAGAGCGGGAGAA-3'
NGQSGR-S	0	0.0	1	0.1	5'-AACGGCCAAAGTGGGAAGGA-3'
NGQSGR-T	0	0.0	1	0.1	5'-AATGGGCAAAGTGGGAGGA-3'
NGQSGR-U	0	0.0	1	0.1	5'-AACGGGCAAAGTGGGAGAA-3'
NGQSGR-X	0	0.0	1	0.1	5'-AACGGGCAAAGTGGGAGAA-3'
NGQSGR-Y	0	0.0	2	0.1	5'-AATGGCCAAAGTGGGAAGAA-3'
NGQSGR-Z	0	0.0	1	0.1	5'-AACGGACAAAGTGGGAAGAG-3'
NGQRGR	0	0.0	7	0.5	5'- AAYGGRCAGCGRGAAGRA -3'
NGQRGR	0	0.0	1	0.1	5'-AACGGACAGCGAGGAAGGA-3'
NGQRGR-AA	0	0.0	4	0.3	5'-AACGGGCAAAGGGGAAGAA-3'
NGQRGR-V	0	0.0	1	0.1	5'-AACGGACAAAGAGGAAGGA-3'
NGQRGR-W	0	0.0	1	0.1	5'-AATGGACAAAGGGGAAGGA-3'
NGQSAR	0	0.0	1	0.1	5'- AACGGACAAAGTGCAAGAA -3'
NGQYGR	0	0.0	1	0.1	5'-AACGGGCAGTATGGGAGAA-3'
NGQNGR	4	2.8	4	0.3	5'- AACGGGCAAATGGGAAGGA -3'
NGRSGR	2	1.4	2	0.1	5'-AACGGGCGAAGTGGGAAGGA-3'
All counts	144		1490		

*Sequences in boldface signify a general protein motif followed by sequence-specific probes for that motif.

negatives + false positives), describes the detection of true H5 sequences against non-target subtypes with similar sequence. Both parameters were calculated by matching the probes to sequences isolated either from humans or, more broadly, isolated from all hosts as shown in Table 1. We are using the term "true" as a probe that contains a homologous sequence to the viral RNA, whereas "false" means no homology. This applies to both positives and negatives. The probe H5G1192 has 100% sensitivity and specificity for subtype identification of H5 in humans at a current limit of detection of approximately 10^8 copies per microliter. In the all host population, the sensitivity decreases to 61.4%. The sensitivity of the H5G1112 probe remains high for cases of human influenza infection at 86.2% but decreases when the target population is expanded to include all hosts to 63.8%. The receptor binding probe, H5R734, has 60% sensitivity for humans and 58.8% sensitivity for all hosts. The cleavage site beacon, H5C1046, has 49% human sensitivity and 42.9% all-host sensitivity. No cross-subtype sequence matches were detected, which is reflected in the high specificity values for the probes. DNA probes containing a hairpin structure exhibit a high specificity for sequence and

discriminate against targets with as few as one nucleotide difference.⁴³

Probe Hybridization to DNA and Synthesized RNA Target

As a control, probes were hybridized to short complementary single-stranded DNA sequences in the absence of secondary structure possible with RNA. The magnesium concentration, present as $MgCl_2$, and temperature were explored for the H5G1112 subtype probe to optimize sequence-specific vRNA detection. The baseline fluorescence signal was corrected by subtracting the measured fluorescence from the probes before the addition of target. At a fixed DNA concentration, the fluorescent signal increased linearly with probe concentration before reaching a plateau at the concentration of probe/concentration of DNA ratio of 1.2. Higher probe concentration ratios produced insignificant increases in fluorescence (data not shown). A thermodynamic response curve was generated for the probe as a function of temperature with and without a complementary DNA target in Figure 2A. The fluorescence of probe H5G1112,

Figure 2. **A:** Thermodynamic response of H5G1112 with and without complementary target DNA at a fixed 6 mmol/L $[\text{Mg}^{2+}]$. The fluorescence is normalized to the probe maximum when hybridized with its DNA complement. Temperature is fixed at 25°C. **B:** The probe, H5G1112, hybridized with complementary target DNA as a function of magnesium concentrations of 6, 16, and 25 mmol/L. The upper trace is 6 mmol/L and the bottom trace is 25 mmol/L $[\text{Mg}^{2+}]$. Temperature is fixed at 25°C. **C:** The maximum fluorescence (squares) of probe H5G1112 and probe hybridization time (circles) as a function of $[\text{Mg}^{2+}]$ at a fixed 25°C temperature when mixed with 45 nmol/L cDNA.

which is shown as a function of magnesium in Figure 2B, increases rapidly in the initial 50 seconds following addition of the DNA target. The maximum fluorescence of the H5G1112 probe hybridized to DNA was inversely proportional to magnesium concentration over the 6 to 25 mmol/L range evaluated (Figure 2C). The hybridization time decreased from 6 mmol/L to 16 mmol/L then reaches a plateau at 25 mmol/L $[\text{Mg}^{2+}]$.

Full-length synthetic H5 influenza viral RNA (1776 bases) and H5G1112 were hybridized in the presence of increasing $[\text{Mg}^{2+}]$ at 25°C as shown in Figure 3A. The normalized fluorescence signal as a function of $[\text{Mg}^{2+}]$ for the H5G1112 probe-vRNA interaction is shown in Figure 3B. The steady-state probe fluorescence when hybridized to vRNA was twofold greater at 25 mmol/L $[\text{Mg}^{2+}]$ compared to 6 mmol/L. The observed fluorescence increased rapidly at low $[\text{Mg}^{2+}]$ and plateaus at 25 mmol/L $[\text{Mg}^{2+}]$, where greater concentrations do not increase the signal further. The rate of probe binding correlated directly with $[\text{Mg}^{2+}]$ to 20 mmol/L. The slope of

the calculated hybridization rate (see Materials and Methods) decreases rapidly past 20 mmol/L then flattens above 50 mmol/L $[\text{Mg}^{2+}]$. The initial hybridization rate was 74 fluorescence units/second in 6 mmol/L $[\text{Mg}^{2+}]$ but was 3.2-fold faster, at 240 fluorescence units/second, with 25 mmol/L $[\text{Mg}^{2+}]$. By increasing the Mg^{2+} content from 6 mmol/L to 50 mmol/L, the computed hybridization time constant, τ , decreased from 620 seconds to only 223 seconds.

It is important to note that we measured four- to 20-fold increase in hybridization with vRNA versus cDNA. This may be due to the fact that the temperature for these reactions was run to optimize signal with vRNA sequences. Within the magnesium concentration ranges and reaction conditions tested here, the interactions with the molecular beacons have favored vRNA secondary structures rather than cDNA structures. Further studies will be necessary to optimize reaction conditions for cDNA sequences and for a common set of reaction conditions to have comparable signals from single-strand RNA and DNA sequences.

Figure 3. H5G1112 subtype probe with vRNA. **A:** The fluorescence response in time of probe H5G1112 mixed with H5 vRNA at increasing concentrations of Mg^{2+} . Temperature was fixed at 25°C. **B:** The maximum fluorescence (squares) of probe H5G1112 and probe hybridization time (circles) as a function of $[Mg^{2+}]$ at a fixed 25°C temperature when mixed with H5 vRNA. **C:** The H5G1112 probe mixed with H5 influenza RNA at multiple temperatures at a fixed 20 mmol/L $[Mg^{2+}]$. At 800s, the fluorescence is ranked in the following order: 45°C, 60°C, 55°C, 37°C, 25°C, 65°C. **D:** The maximum fluorescence (squares) of probe H5G1112 and probe hybridization time (circles) as a function of temperature at a fixed 25 mmol/L $[Mg^{2+}]$ when mixed with H5 vRNA. **E:** The detection limit of vRNA is 0.61 nmol/L or 4×10^8 molecules/ μ L in 30 nmol/L of H5G1112 and 25 mmol/L $[Mg^{2+}]$. The fluorescence value at 300 seconds was used in the calculations.

Probe H5G1112 behavior with H5 vRNA was characterized as a function of temperature at 25 mmol/L $[Mg^{2+}]$ in Figure 3C. Both the hybridization rate and the fluorescence maximum are shown in Figure 3D as a function of temperature. The hybridization rate decreased linearly with increasing temperature. The maximum fluorescence reaches a plateau at 45°C to 55°C and dropped rapidly at higher temperatures. This observed steady-state fluorescence showed a 40% greater signal at 55°C over 25°C.

The probe H5G1192 is an additional subtype probe that can identify 100% of the H5 viruses isolated thus far

from humans and sequenced in NCBI. Since this probe binds a location that differs from the H5G1112 subtype probe, secondary structure of RNA is expected to modify the probe hybridization profile. The hybridization characteristics of H5G1192 with its complementary DNA probe indicated 45°C was a preferred temperature (data not shown). Figure 4A shows the H5G1192 probe hybridizing to H5 vRNA at 45°C at several concentrations of Mg^{2+} . The hybridization time decreases linearly with Mg^{2+} concentration in Figure 4B. The maximum fluorescence increases slightly up to 50 mmol/L $[Mg^{2+}]$ but increases rapidly at 75 mmol/L.

Figure 4. H5G1192 subtype probe. **A:** The fluorescence response in time of probe H5G1192 mixed with H5 vRNA at increasing concentrations of Mg^{2+} . Temperature was fixed at 45°C. **B:** The maximum fluorescence (squares) of probe H5G1192 and probe hybridization time (circles) as a function of $[Mg^{2+}]$ at a fixed 25°C temperature when mixed with 45 nmol/L H5 vRNA.

The sialic acid receptor probe, H5R734, and the hemagglutinin cleavage motif probes, H5C1045 and H5C1046, were hybridized with H5 vRNA in Figure 5. The hybridization rate for probe H5C1045 is 2.5 times greater than for probe H5C1046. The specificity of the H5G1112 probe, shown in Figure 6, was evaluated experimentally with complementary

Figure 5. Fluorescence response in time of H5C1045 (upper trace), H5C1046 (lower trace) cleavage probes and H5R734 (middle trace at 1000 seconds) receptor probe when mixed with 45 nmol/L H5 vRNA at 25 mmol/L $[Mg^{2+}]$. Temperature was fixed at 25°C.

Figure 6. The effect of point mutations on the fluorescence of probe H5G1112 as a function of temperature at a fixed 25 mmol/L $[Mg^{2+}]$. The fluorescence values at 300 seconds were used.

DNA containing one of five point mutations present in the false negative H5 population of this probe. These DNA nucleotide point mutations listed by count and location in the probe sequence are shown in Table 4 as the reverse complement, which correspond to the NCBI database of cDNA.

Each of these DNA single point mutation targets confirmed that the probes have a high specificity for sequence recognition under the proper conditions. At 55°C, the fully complementary cDNA sequence maintained the fluorescence from six to 21 times greater than single point mutations. Fully mismatched targets, which are not shown, do not increase fluorescence above the baseline fluorescence of probe without target, as shown in Figure 2A.

Minimum Detectable vRNA Concentration

We have defined the signal-to-noise ratio as $(\text{fluorescence}_{\text{probe + target}})/(\text{fluorescence}_{\text{probe only}})$. A signal-to-noise ratio of 3, our chosen threshold, indicates that the fluorescent signal is 3 SD (99.7% confidence) above the baseline noise. The minimum detectable concentration is calculated as $3 \times \text{noise/signal}$, where noise (fluorescence units/nM probe) and signal (fluorescence units/nM vRNA/nM probe) are measured quantities. The minimum detectable concentration of H5G1112 probe and vRNA in the fluorometer (see Materials and Methods) was evaluated at 55°C and 25 mmol/L $[Mg^{2+}]$. Fluorescence was also measured at multiple concentrations of vRNA, while maintaining a constant concentration of probe to concen-

Table 4. cDNA Point Mutation Targets

Probe mutation	Sequence*
H5G1112 non-mutant	5'-ATGGTAGATGGTTGGTATGGGTA-3'
H5G1112-G7C	5'-ATGGTACATGGTTGGTATGGGTA-3'
H5G1112-T9C	5'-ATGGTAGACGGTTGTATGGGTA-3'
H5G1112-T12C	5'-ATGGTAGATGGCTGGTATGGGTA-3'
H5G1112-T16C	5'-ATGGTAGATGGTTGGCATGGGTA-3'
H5G1112-G21T	5'-ATGGTAGATGGTTGGTATGGTTA-3'

*Boldface indicates point mutation.

tration of RNA ratio of 1.2. The fluorescence signal responded at a rate of 2778 ± 28 (fluorescence units/nM vRNA/nM probe), whereas fluorescence in the absence of RNA target, defined as noise, increased 368 ± 4 (fluorescence units/nM probe). Using the subtype identification probe, H5G1112, the minimum detectable H5 vRNA concentration at a limiting condition of a signal to noise ratio of 3 is 0.4 ± 0.02 nmol/L ($\sim 1.2 \times 10^8$ copies/ μ l). Similarly, the subtype probe, H5G1192, has a sensitivity limit of 0.6 ± 0.02 nmol/L ($\sim 1.8 \times 10^8$ copies/ μ l) at $T = 45^\circ\text{C}$ and 25 mmol/L magnesium concentration.

Discussion

Molecular beacons were developed for sequence-specific direct detection of the hemagglutinin segment of H5 influenza RNA with no thermocycling. The optimal conditions for hybridization of these influenza probes to viral RNA are fundamentally different from the optimal binding conditions to target DNA, as in a reverse transcriptase-polymerase chain reaction application. Figure 2 shows the effects of temperature and concentration of Mg^{2+} on thermodynamic base pairing, which was first tested by hybridizing short complementary DNA to the H5G1112 probe. Without target, the fluorescence in Figure 2A remains minimized until reaching the probe T_m at 55°C . Above this, the probe opens and unquenches but adopts a random coil structure, which limits the fluorescence to 20% of maximum. Probe-vRNA binding at temperatures below the T_m produces a maximum fluorescent signal, which also transitions to a low fluorescence random coil state above the melt temperature. A 6 mmol/L [Mg^{2+}] formulation produces the highest fluorescent signal with a response time only 13% slower than with higher concentration Mg^{2+} formulation as shown in Figure 2C. The fluorescence decreased twofold as concentration of Mg^{2+} increased from 0 to 25 mmol/L but remained constant (data not shown) over the temperature range of 25°C to 55°C . The increasing magnesium concentration thermodynamically disfavors base pairing of the molecular beacons and complementary DNA.

In contrast to DNA targets, the H5G1112 probe-vRNA interactions provided in Figure 3 are enhanced by [Mg^{2+}]. The detection efficiency of H5 vRNA depends on the secondary structure of RNA as suggested in Figure 3B by the effects of magnesium concentration on the probe response. Fluorescence increases and the calculated probe hybridization/response time, τ , decreases such that 25 mmol/L [Mg^{2+}] appears optimal. The second subtype probe, H5G1192, shown in Figure 4 responds similarly to H5G1112 to magnesium and temperature in that hybridization time decreases and fluorescence increases with increasing magnesium. H5G1192 does not exhibit large increases in fluorescence until exceeding 50 mmol/L Mg^{2+} . Concentrations above 75 mmol/L, which exhibited the highest fluorescence of conditions tested, were not evaluated but may exhibit even greater fluorescence. A hybridization time minima may exist also past 75 mmol/L. The probe binding location, which is shifted by 90 nucleotides to the 3' end of a cDNA reference, modifies the hy-

bridization kinetics. The H5G1192 general H5 subtype probe, which identifies 100% of the human H5 strains, is twice as slow to respond at 25 mmol/L Mg^{2+} than the H5G1112 general H5 subtype probe under the respective test conditions. The receptor probe, H5R734, and hemagglutinin cleavage probes H5C1045 and H5C1046 each exhibit different hybridization response times (Figure 5).

Stable secondary and tertiary structures of single-strand vRNA, which may obstruct the target sequence, regulate the probe hybridization efficiency. MFOLD simulations (<http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/ma-form1.cgi>)^{44,45} show that the 1776-base length H5 vRNA can adopt multiple long-lived conformations, which determine the binding sequence availability and therefore the response time of the probe. Although the probes used in this study selectively bound influenza vRNA, the observed hybridization rate of the probe with vRNA was much slower than with short complementary DNA oligomers. This suggests that conformational switching of influenza RNA structure is a rate limiting step. Enhanced temperature at 25 mmol/L [Mg^{2+}] disrupt the secondary structure to increase the rate of hybridization with vRNA as summarized in Figure 3D. Consequently, this suggests that secondary structure of the vRNA at low concentration of Mg^{2+} inhibits access of the probe to its target. On the contrary, previous studies on ribosomal RNAs found that [Mg^{2+}] increased the compaction or folding.^{21,46} Hence, it appears that the interaction between magnesium ions and influenza vRNA molecules is fundamentally different from those observed between [Mg^{2+}] and ribosomal RNAs.

The effect of point mutations on the fluorescent response of the H5G1112 subtype probe were evaluated using DNA oligos with single point substitutions as shown in Figure 6. These mutations were selected to match mutations found in the wild-type population of H5 virus. The point mutation lowers the T_m of the probe-target hybrid. Below 50°C , the mismatched probes will partially hybridize and produce fluorescence less than half of the perfect target. Completely mismatched targets have no significant impact on fluorescence. At 55°C , only the perfectly matched probe maintains a high fluorescence, whereas the other mismatched combinations transition to a random coil state with quenching. Due to the stronger interaction potential of RNA:DNA hybrids, we expect even higher specificity with viral RNA variants compared to the DNA:DNA mutants of Figure 6.

To our knowledge, this is the first study quantifying the effect of concentration of Mg^{2+} on the secondary structure of H5 influenza RNA. Direct probing of RNA *in vivo* faces the additional challenges of nuclease contamination, which threatens the stability of both DNA probes and RNA targets.⁴⁷ H5 viral extract was not conducted due to accessibility and biohazard concerns. The vRNA present in active infection or encapsidated in viral particles is bound by viral nucleoproteins, which can reasonably be expected to influence the secondary structure of vRNA. Viral RNA purification, which neutralizes nucleases and removes proteins associated with vRNA, is standard procedure for diagnostic testing. The *in vitro* transcribed

vRNA evaluated here is similarly without viral protein cofactors.

The specificity and sensitivity of viral RNA detection probes are critical performance parameters for a diagnostic hybridization assay. For humans, the H5G1112 probe target sequence produced 20 false negative results when queried against the NCBI database. However, these 20 records were taken from eight individuals each with a single infection. In some cases, the NCBI records showed that sampling occurred over a multi-day span or was collected on the same day using different techniques, such as by an endotracheal tube throat wash or nasal swab. Other sequences differed by a single ambiguous base, possibly from sequencing error, at distal points far from the target region of the probe but were included as two separate samples. Overall 60% of the false negatives could be considered redundant. Since this same sample bias potentially exists with our positive samples as well, no data points were excluded nor did we restrict analysis only to full-length sequences. Phylogenetic analysis was further restricted to coding regions of which only 105 were available in the database. Based on these known H5 mutations, additional DNA probes could be synthesized to detect these H5 point mutants and boost the sensitivity while maintaining specificity. As one exception, a G21T mutation in the H5G1112 target sequence would include detection of 42 H1N1 strains as false positives out of 4287 true negatives and would reduce specificity to 99%.

For each sequence target, the hybridization times to achieve a signal to noise ratio of 3 may vary over several hundred seconds. These times are based on constant temperature and counter ion conditions. To normalize the fluorescent signal intensity among wells, the RNA can be heated above the melt temperature of molecular beacons and then decreased to 55°C to regain specific signal. The heat drives the thermodynamic equilibrium toward binding by exposing binding sites in the RNA to the beacon. A thin film of indium–tin oxide has been used successfully to heat a specific printed pattern with high optical transmissibility and low power consumption in a wide range of devices.⁴⁸ Fluorescent DNA molecular beacons were selected for their self-quenching property over colorimetric probes. These enzymatic colorimetric reactions currently cannot be switched off and on, which eliminates the ability to discriminate single base pair mutations. Fluorescence can be stimulated using blue/ultraviolet light-emitting diodes and detected with an inexpensive photon detector.^{49–53}

It is important to note that this work relates to the analytical sensitivity of the H5 vRNA, and there is no attempt made to compare the detection sensitivity with the currently available diagnostic tests that are being tested on clinical samples. Furthermore, the assay described in this manuscript is only a part of the overall detection system planned, ie, the probes are to be coupled with an isothermal amplification and are not guaranteed to detect nonamplified viral RNA in clinical samples.

Conclusion

Rapid determination of pathogenic viral risk will improve the public health response to emerging pandemic influenza. A direct, non-thermocycled method of extracting sequence-specific information from RNA was investigated using viral RNA from a highly pathogenic H5 species and dual-labeled, fluorescent DNA probes for detection. Direct subtype identification of full-length RNA requires experimental conditions that are distinctly different from those for short DNA segments. Secondary and tertiary structures dominate the probe response. The hemagglutinin segment of the H5 subtype was surveyed for three parameters, which help characterize the viral strain as highly pathogenic or low pathogenic. Probe sequences that could identify the general H5 subtype, detect the avian $\alpha 2,3$ or human $\alpha 2,6$ sialic acid binding preference, and probe the cleavage motif, where polybasic acids are characteristic of high pathogenic avian influenza were found. The general probes H5G1112 and H5G1192 detected *in vitro* transcribed RNA from a A/Hanoi/30408/2005(H5N1) template down to 0.4 ± 0.02 nmol/L (at $T = 55^\circ\text{C}$) and 0.6 ± 0.02 nmol/L (at $T = 45^\circ\text{C}$), respectively, under the limiting conditions of a signal-to-noise ratios of 3. The probe demonstrated high specificity for sequence near the probe T_m using point mutant DNA targets. The results showed almost no increase in the fluorescence above a signal to noise ratio of 3 at $T = 55^\circ\text{C}$. The hybridization conditions of Mg^{2+} concentration, temperature, and time defined the fluorescence response of the probe to vRNA. Although the current limits of detection for probes was suboptimal, the results demonstrate that molecular beacons are suitable for detection of vRNA despite the challenges of secondary structure.

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