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ADAPTING THE EURASIAN H5 TAQMAN RT-QPCR ASSAY FOR THE DETECTION OF AVIAN INFLUENZA STRAINS CIRCULATING IN INDIA

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Abstract – The development of molecular diagnostic for avian influenza virus (AIV) is a continuous process due to evolution of virus after each passing outbreak. The molecular tests need to be reviewed after each outbreak for obtaining consistent laboratory results. In the present study, the oligos sequences of validated H5 Eurasian taqman RT-qPCR for H5 subtyping have been improved matching to the currently circulating strains for laboratory diagnosis of AIV. All available influenza H5 hemagglutinin gene sequences of Eurasian strains from the public databases were aligned in-silico with the recommended RT-qPCR oligos and modifications were done according to the matching sequences of the recently evolved strains. The modified oligos were validated with clinical samples and reference AI H5N1 isolates belonging to different clades. Multiple sequence alignment suggested the modifications at two, three and one positions respectively in probe, forward and reverse primers. Out of 1295 clinical samples tested, 251 samples were found positive. Assay could identify all the 115 virus strains of AI H5 subtype belonging to clade 2.2, 2.2.2, 2.3.2.1C, 2.3.2.1a and 2.3.4.4b. On comparison to the virus isolation, the positive and negative agreement was found to be 0.925 ± 0.025 and 0.983 ± 0.002 respectively. Analytical specificity of the modified assay was confirmed specifically with AI H5 subtype only whereas analytical sensitivity was found to be 13-130 copies of IVT-RNA and 0.1 EID_{50} of AI H5N1 virus. We propose that the modified RT-qPCR assay can be a choice for routine diagnosis of Eurasian strains of AI H5 subtypes.

INTRODUCTION

Over a period of more than two decades, influenza H5N1 viruses have emerged and diverged into many clades and subclades. Till date more than 68 countries have faced the outbreaks of AI H5N1 infection all over the world. During the spread of infection, particularly Eurasian strains of these viruses have further evolved into much diverged form (WHO/OIE/FAO H5N1 Evolution Working Group, 2012 and 2014). The diagnosis of avian influenza by conventional technique of virus isolation is a time taking protocol and requires handling of procedure into strict bio-containment setup. Alternatively, molecular test used for the diagnosis including the conventional reverse transcriptase polymerase chain reaction (RT-PCR) and realtime reverse transcriptase polymerase chain reaction (RT-qPCR) are although sophisticated but produce results within few hours with higher sensitivity. Presently many protocols of RT-qPCR for H5 subtyping are available and few of them have also been recommended by OIE for the diagnosis (typing and subtyping) of avian influenza viruses (Chen *et al.,* 2015; OIE, 2024; Spackman *et al.,* 2002; Slomka *et al.,* 2007).

In the present study, validated H5 Eurasian taqman RT-qPCR for avian influenza H5 subtyping test, primarily reported by *Spackman et al.,* (2002); and thereafter modified by Slomka *et al.,* (2007) has been reviewed and modified as per the presently available sequences of HA-H5 Eurasian strains with special preference to submission of gene bank sequence by SAARC countries. The modified assay was evaluated for analytical and diagnostic performance and validated with true clinical samples.

MATERIALS AND METHODS

Avian Influenza Virus strains: Avian influenza viruses used in the present study (Table 1), were taken in form of allantoic fluid from the repository of ICAR-National institute of High Security Animal Diseases (ICAR-NIHSAD), Bhopal, India. The viruses were propagated in approximately 9-11 days old embryonated chicks for 2-7 days or until death of embryos at 37^{0}C as per the standard protocol in Biosafety Level 3 (BSL3) facility. The isolation of virus in allantoic fluid was confirmed by the standard procedure of Hemagglutination (HA) and Hemagglutination Inhibition (HI) tests.

Field/clinical samples: The field/clinical samples used in the study comprised of carcasses, cloacal, fecal, tracheal and oropharyngeal swabs, tissues, fecal materials, mud/water samples etc. (Table 2) were received from the field and zoological parks during the outbreaks or emergency situation with high morbidity and mortality in India. The samples comprised of black-necked stork (*Ephippiorhynchus asiaticus*) - 01, brahminy kite (*Haliasturindus*) - 01, poultry - 563, cockatiel (*Nymphicus hollandicus*) -02, crane bird - 02, domestic crow - 39, domestic duck - 226, fowl/ guinea Fowl - 99, giriraja - 01, domestic goose (*Anserdomesticus*) - 12, great white pelican (*Pelecanusonocrotalus*) - 10, herons - 01, munia (*Lonchurapunctulata*) - 03, painted stork (*Mycterialeucocephala*) - 21, peacock/peafowl (*PavoCristatus*) - 02, pigeon - 07, silver pheasant (*Lophu ranycthemera*) - 01, turkey - 05, zebra finch (*Taeniopygia guttata*) - 01, others species - 277, mud and water samples -21.

Alignment of nucleotide sequence and modifications in oligos: Total 2585 nucleotide sequences of HA5 gene submitted during 2007-2018 for Eurasian strains available through NCBI, Fludb, and gisaid databases were aligned in multiple sequence alignment format (Bio Edit version 7.2.5) and binding sites of primers and probe reported by Slomka *et al.* (2007) were determined for variation in nucleotides for each position. Accordingly, modifications were done in existing primers and probe and their efficiency was evaluated statistically for G+C content, primer-dimer formation, secondary structure and hairpin formation etc. using online tools. The modified probe was synthesized with FAM reporter and BHQ1 quencher dye.

RNA isolation: Viral RNA was isolated from the 140µl of allantoic fluid or processed clinical materials as per the manufacturer's protocol using QIAamp viral RNA or RNeasy Mini Kit (QIAGEN, Germany). The RNA was eluted in 60 µl of elution buffer and stored at -70^{0}C until used.

One Step Taqman Real Time reverse transcriptase PCR (RT-qPCR): RT-qPCR was carried out in duplicates for each samples using superscript III platinum one step Quantitative RT-PCR System (Cat No. 11732-020, Invitrogen) in a total volume of 12.5 µl containing 6.25 µl of 2x buffer, 10 pmol of each forward and reverse primer, 5 pmol of probe, 1.25 U of superscript III RT/Platinum Taq Mix, and 2µl of viral RNA template. The PCR assay was conducted on Roche Light Cycler® 480 II System. The thermal profile was programmed as 30 min at 50 ^{0}C, 5 min at 95^{0}C followed by 40 cycles of 3 step PCR (30 sec at 95^{0}C, 60 sec at 50 ^{0}C and 30 sec at 72 ^{0}C). The fluorescence reading was acquired at the end of each extension step. No template control (NTC) and no probe control (NPC) was also included in every run.

Determination of analytical sensitivity, specificity and assay precision: To determine the sensitivity of RT-qPCR, a full length standard DNA clone of HA5 gene (accession number EPI106676) in pGEM-T easy vector (Promega, USA) was linearized using Nco I restriction enzyme and *in-vitro* RNA was transcribed using SP6 polymerase as per the manufacturer's protocol (RiboMAX Large Scale RNA Production System-SP6, Promega). The resulting in-vitro transcribed RNA (IVT-RNA) was purified and quantified. The copy number was accordingly calculated using the following standard formula

RNA copies per microliter

$$= \left[\frac{Amount\ of\ RNA\ in\ gram\ per\ microliter}{\text{transcript length in nucleotides}} \times 340\right] \times 6.022 \times 10^{23}$$

The IVT-RNA was 10 fold serially diluted ranging from 1.3x10^8 to ≤1.3 copies/µl and 1 µl of IVT-RNA from each dilution was subjected to RT-qPCR assay in triplicate for preparation of standard curve and determination of analytical sensitivity. Mean standard deviation (SD) and coefficient of variation (CV) was calculated for each dilution with respect to Cp (cross-point cycle) values. The variability (CV) was expressed as $\frac{SD}{Mean} \times 100$. Similarly, AI H5N1 virus with known EID_{50} titer was 10 fold serially diluted in uninfected allantoic fluid from specific pathogen free (SPF) eggs to determine the analytical sensitivity in terms of EID_{50}. The RNA extracted

from each dilution was thereafter used for preparation of standard curve.

The analytical specificity of the assay was evaluated by cross-reaction test with RNA extracted from other AIV subtype (H1-H16) and Newcastle disease virus (NDV).

Assessment of diagnostic performance of modified RT-qPCR with clinical samples and AI H5 reference viruses: A total of 1295 field/clinical samples (Table 2) were tested for virus isolation and by modified RT-qPCR assay. In addition, 115 AI H5 reference viruses belonging to different clades were tested in RT-qPCR to assess the diagnostic scope of the modified assay (Table 1).

Determination of agreement between RT-qPCR and Virus isolation tests: The agreement between modified RT-qPCR and virus isolation was calculated using the following formula (Cicchetti and Feinstein, 1990).

$$Positive\ agreement = \frac{2a}{2a + b + c}$$

$$Negative\ agreement = \frac{2d}{2d + b + c}$$

Calculation for standard error was done according to Mackinnon (2000).

$$Standard\ error\ for\ positive\ agreement = \sqrt{[4a(c+b)(a+c+b)]}/(2a+b+c)2$$

$$Standard\ error\ for\ negative\ agreement = \sqrt{[4d(c+b)(d+c+b)]}/(2d+b+c)2$$

a – number of samples positive by both X and Y tests; **b** – number of samples positive by X test and negative by Y test; **c** – number of samples negative by X test and positive by Y test; **d** – number of samples negative by both X and Y tests. Virus isolation was taken as reference test for determining the agreement between two tests.

RESULTS AND DISCUSSION

Since 2006, India has consistently faced annual outbreaks of AI H5 subtype in poultry, ducks, domestic, and wild birds. The AI H5 viruses responsible for these outbreaks belong to clades 2.2 (Chakrabarti *et al.*, 2009; Pattnaik *et al.*, 2006; Tosh *et al.*, 2011), 2.2.2, 2.3.2.1C, 2.3.2.1A (Nagarajan*et al.*, 2011; Tosh *et al.*,2016) and 2.3.4.4B (Nagarajan *et al.*,2017), indicating frequent viral evolution and diversification. Molecular diagnostic tests, specifically Taqman RT-qPCR, play a crucial role in early detection and control of avian influenza outbreaks. Given the rapid evolution of AI H5 viruses, it is imperative that molecular assays be periodically updated to maintain their diagnostic accuracy and sensitivity.

Among the most trusted and widely used tests for AI H5 subtyping is the assay developed by Slomka *et al.*, (2007), which has been validated by numerous international reference laboratories. However, this assay was based on Eurasian AI H5 strains circulating in 2007, and given the significant divergence of H5 strains over the past two decade, there was a need to transform the assay to align with current sequences. The present study was undertaken to improve the H5 Taqman RT-qPCR assay in light of recent outbreaks, especially in the SAARC region, using sequences from flu databases. The modifications were made to avoid false negative results due to viral mutations affecting primer and probe binding sites. Approximately 2585 HA5 sequences, submitted after 2008, were analyzed for variations at the binding sites of the primers and probe. The modifications included the addition of degenerate bases at two positions in the probe and changes at three positions in the forward primer and one position in the reverse primer (Table 3). The quencher was also replaced with a non-fluorescent black hole quencher (BHQ1) to reduce background signal. Validation of the modified oligonucleotides was confirmed through the successful generation of a sigmoid amplification curve in the RT-qPCR assay.

Many studies underscore the need for precise primer design, which informed our decision to modify the assay to enhance its accuracy across different avian species. These adjustments mirror the Tseng *et al.* (2014) methodology, which described a complete molecular diagnostic procedure used for surveillance and subtyping of avian influenza virus. Their findings, along with recommendations from WHO (2011, 2014, 2018), highlight the importance of

Table 2. Clinical samples tested by RT-qPCR and Virus isolation (VI)

Samples	Tested	Positive	
		RT-qPCR	VI
Carcass	187	152	139
Cloacal Swabs	495	28	22
Fecal droppings	38	01	01
Fecal Swabs	146	21	14
Mud/Water sample	21	00	00
Oropharyngeal swab	02	00	00
Tracheal Swabs	315	22	20
Tissue	91	27	24
Total	**1295**	**251**	**220**

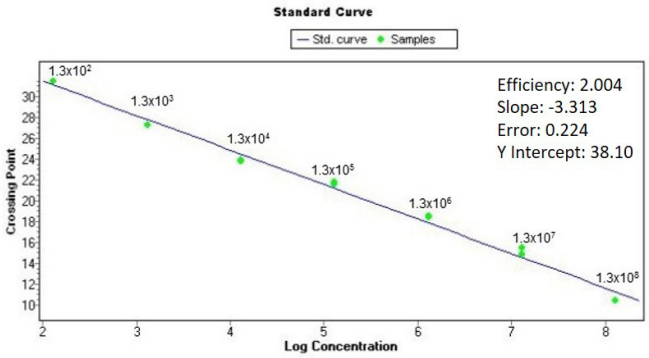

Fig. 1. **Determination of Analytical sensitivity:** A standard plot was generated using AI H5-HA gene specific IVT-RNA ranging from $1.3x10^8$ to 3 copies. The analytical sensitivity was evaluated upto 13 copies. However linearity of the curve was observed upto $1.3x10^2$ copies.

adapting assays to meet evolving viral sequences and epidemiological contexts.

The modified assay was tested for specificity using RNA from AI H1 to H16 subtypes and Newcastle disease virus (NDV), confirming specificity for AI H5 only. The analytical sensitivity of the assay was determined using IVT-RNA, with a limit of detection (LOD) 13-130 copies of IVT-RNA. The assay demonstrated linearity between 1.3×10^8 and 1.3×10^2 copies of IVT-RNA with 100% PCR efficiency (Figure 1 and Table 4). The mean standard deviation (SD) and coefficient of variation (CV%) for Cp values were ±0.16 and 0.82%, respectively. The assay's analytical sensitivity is consistent with previous studies (Li*et al.,* 2008, Payungporn *et al.*,2006; Suwannakarn *et al.*,2008; Wu *et al.,* 2008), although it significantly improved upon the original assay's sensitivity (Spackman *et al.,* 2002).

The assay also demonstrated a detection threshold of 0.1 EID_{50} for AI H5 subtype virus, similar to the original protocol. The clinical cutoff, based on both analytical sensitivity and diagnostic performance, was determined to be Cp-35. Among 1295 clinical samples tested, the modified RT-qPCR assay detected 251 positives, while virus isolation yielded 220 positives (Table 5). Two samples that were virus isolation-positive but assay-negative were later confirmed positive using allantoic fluid from virus isolation, indicating that the discrepancy may have been due to PCR inhibitors or poor sample conditions.Virus isolation was employed as the gold standard reference test for analysis. However, the virus could not be isolated in 33 samples that tested positive by RT-qPCR, likely due

Table 4. Determination of analytical sensitivity using H5 IVT-RNA with average Cp (Cp avg.), Standard deviation (SD) and Coefficient of variation (CV %)

Copies	Cp avg.	SD	CV (%)
$1.3x10^8$	10.40	0.03	0.29
$1.3x10^7$	15.13	0.43	2.84
$1.3x10^6$	18.41	0.09	0.49
$1.3x10^5$	21.66	0.17	0.78
$1.3x10^4$	23.78	0.08	0.34
$1.3x10^3$	27.26	0.20	0.73
$1.3x10^2$	31.45	0.09	0.29

Table 5. Comparison of test results for virus isolation (VI) and RT-qPCR test for clinical samples

	RTqPCR		
	Positive	Negative	**Total**
VI Positive	218	02	220
VI Negative	33	1042	1075
Total	**251**	**1044**	**1295**

Table 3. Binding sites for the sequence (5'to 3') of hydrolysis probe and primers for H5 subtyping

Study	Probe	Forward primer	Reverse primer
Slomka *et al.* (2007)	TCWACAGTGGCGAGTTC CCTAGCA	ACATATGACTACCCA CARTATTCAG	AGACCAGCTAYCAT GATTGC
Eurasian P-11 (Present study)	TCWACAGTGGCG AG**Y**TCCCTAG**Y**A	AC**G**TATGACTACCC**G**C A**G**TATTCAG	AGACCAGC**Y**AYCATG ATTGC

* The bold and underlined nucleotide bases are position for modification

to loss of infectivity during sample transport or low viral load in the clinical samples. Additionally, all 33 samples that were negative by virus isolation showed Cp values >32, further suggesting a low viral load in these samples.

Our results showed significant positive and negative agreements with virus isolation (0.925 ± 0.025 and 0.983 ± 0.002, respectively). The assay successfully detected a panel of 115 AI H5N1 viruses belonging to clades 2.2, 2.2.2, 2.3.2.1C, 2.3.2.1A, and 2.3.4.4B, confirming its broad applicability (Table 1).

Although the original assay developed by Slomka *et al.,* (2007) remains gold standard, the modifications we propose offer an updated option for diagnostic laboratories, ensuring that the assay maintains its diagnostic potential for a wide range of AI H5 strains, especially those currently circulating. This modified assay has been rigorously validated, including tests for analytical and diagnostic sensitivity and specificity. While the modified assay has not been parallelly tested with the original assay on all clinical samples, we believe the modifications offer an enhanced diagnostic tool aligned with

Table 1. List of AI H5 subtype reference viruses used for the validation of modified RT-qPCR assay

SN	Strain	Host	GISAID accession	Clade
1	A/chicken/Navapur/7972/2006	Chicken	EPI225017	2.2
2	A/chicken/Jalgaon/8824/2006	Chicken	EPI106676	2.2
3	A/chicken/Nandurbar/7979/2006	Chicken	EPI354408	2.2
4	A/chicken/Nandurbar/7966/2006	Chicken	EPI306395	2.2
5	A/chicken/Jalgaon/12419/2006	Chicken	EPI163702	2.2
6	A/chicken/Jalgaon/13732/2006	Chicken	EPI163694	2.2
7	A/chicken/Jalgaon/13735/2006	Chicken	EPI163686	2.2
8	A/chicken/Manipur/NIV9743/2007	Chicken	EPI174900	2.2
9	A/chicken/West Bengal/81010/2008	Chicken	EPI184277	2.2.2.1
10	A/chicken/West Bengal/80995/2008	Chicken	EPI184274	2.2.2
11	A/chicken/West Bengal/82544/2008	Chicken	EPI184255	2.2.2
12	A/chicken/West Beng al/82583/2008	Chicken	EPI352513	2.2.2
13	A/chicken/West Bengal/81537/2008	Chicken	EPI352505	2.2.2
14	A/chicken/West Bengal/81760/2008	Chicken	EPI287579	2.2.2
15	A/chicken/India/81754/2008	Chicken	EPI438838	2.2.2
16	A/chicken/India/81753/2008	Chicken	EPI438830	2.2.2
17	A/chicken/India/81766/2008	Chicken	EPI372255	2.2.2
18	A/chicken/India/81890/2008	Chicken	EPI438846	2.2.2
19	A/chicken/India/82544/2008	Chicken	EPI438857	2.2.2
20	A/chicken/India/82580/2008	Chicken	EPI438865	2.2.2
21	A/chicken/India/82616/2008	Chicken	EPI438873	2.2.2
22	A/chicken/West Bengal/82613/2008	Chicken	EPI287576	2.2.2
23	A/chicken/India/83093/2008	Chicken	EPI438882	2.2.2
24	A/chicken/West Bengal/83098/2008	Chicken	EPI352521	2.2.2
25	A/chicken/India/85459/2008	Chicken	EPI438890	2.2.2
26	A/chicken/India/96880/2008	Chicken	EPI438894	2.2.2
27	A/chicken/India/100071/2008	Chicken	EPI372239	2.2.2
28	A/chicken/West Bengal/100879/2008	Chicken	EPI287577	2.2.2.1
29	A/duck/Tripura/103597/2008	Duck	EPI295967	2.2.2.1
30	A/goose/Tripura/103596/2008	Goose	EPI295959	2.2.2.1
31	A/duck/India/TR-NIV4396/2008	Duck	EPI221341	2.2.2.1
32	A/chicken/West Bengal/106181/2008	Chicken	EPI287578	2.2.2.1
33	A/chicken/Assam/140203/2008	Chicken	EPI175658	2.2.2.1
34	A/chicken/Assam/140187/2008	Chicken	EPI175656	2.2.2.1
35	A/crow/Assam/142119/2008	Corvus	EPI255774	2.2.2.1
36	A/chicken/West Bengal/142121/2008	Chicken	EPI287624	2.2.2
37	A/chicken/Assam/142007/2008	Chicken	EPI291945	2.2.2.1
38	A/chicken/West Bengal/148713/2009	Chicken	EPI287631	2.2.2
39	A/chicken/West Bengal/155505/2009	Chicken	EPI287607	2.2.2.1
40	A/chicken/Sikkim/151464/2009	Chicken	EPI354416	2.2.2

41	A/chicken/Sikkim/151466/2009	Chicken	EPI352481	2.2.2
42	A/chicken/West Bengal/155508/2009	Chicken	EPI352489	2.2.2.1
43	A/chicken/West Bengal/162642/2009	Chicken	EPI287575	2.2.2
44	A/chicken/West Bengal/170564/2009	Chicken	EPI287574	2.2.2
45	A/chicken/West Bengal/174881/2009	Chicken	EPI352497	2.2.2
46	A/chicken/West Bengal/193936/2009	Chicken	EPI287615	2.2.2
47	A/chicken/West Bengal/239022/2010	Chicken	EPI287692	2.2.2.1
48	A/chicken/West Bengal/239020/2010	Chicken	EPI287684	2.2.2
49	A/chicken/India/241272/2010	Chicken	EPI372247	2.2.2
50	A/chicken/Bhutan/248006/2010	Chicken	EPI336725	2.2.2
51	A/chicken/Bhutan/248007/2010	Chicken	EPI336717	2.2.2
52	A/chicken/Bhutan/248015/2010	Chicken	EPI317301	2.2.2.1
53	A/chicken/Bhutan/248009/2010	Chicken	EPI317293	2.2.2
54	A/duck/India/02AF1/2011	Duck	EPI352287	2.3.2.1A
55	A/duck/India/02CA10/2011	Duck	EPI352310	2.3.2.1A
56	A/chicken/India/03CL488/2011	Chicken	EPI471860	2.3.2.1A
57	A/chicken/India/TR0383/2011	Chicken	EPI352325	2.3.2.1A
58	A/chicken/India/CA0302/2011	Chicken	EPI352323	2.3.2.1A
59	A/chicken/India/CA0301/2011	Chicken	EPI352321	2.3.2.1A
60	A/chicken/India/CL03485/2011	Chicken	EPI352318	2.3.2.1A
61	A/chicken/India/CA0303/2011	Chicken	EPI352315	2.3.2.1A
62	A/chicken/India/09CA09/2011	Chicken	EPI542201	2.3.2.1A
63	A/chicken/India/09CA02/2011	Chicken	EPI542198	2.3.2.1A
64	A/chicken/India/09CA10/2011	Chicken	EPI472245	2.3.2.1A
65	A/chicken/India/09CA01/2011	Chicken	EPI472243	2.3.2.1A
66	A/crow/India/11TI07/2011	Corvus	EPI623582	2.3.2.1A
67	A/crow/India/11CA01/2011	Corvus	EPI585446	2.3.2.1A
68	A/crow/India/11TI16/2011	Corvus	EPI642493	2.3.2.1A
69	A/crow/India/11TI11/2011	Corvus	EPI642463	2.3.2.1A
70	A/crow/India/11CL01/2011	Corvus	EPI585453	2.3.2.1A
71	A/crow/India/01TR01/2012	Corvus	EPI558719	2.3.2.1A
72	A/chicken/India/01CA01/2012	Chicken	EPI685614	2.3.2.1A
73	A/chicken/Bhutan/01TR12/2012	Chicken	EPI644490	2.3.2.1A
74	A/chicken/Bhutan/01CL140/2012	Chicken	EPI644483	2.3.2.1A
75	A/crow/India/01CA10/2012	Corvus	EPI621152	2.3.2.1A
76	A/crow/India/01CA08/2012	Corvus	EPI621148	2.3.2.1A
77	A/duck/India/01CA01/2012	Duck	EPI685610	2.3.2.1A
78	A/crow/India/01CA12/2012	Corvus	EPI644411	2.3.2.1A
79	A/chicken/India/01CA06/2012	Chicken	EPI585428	2.3.2.1A
80	A/chicken/India/01CA03/2012	Chicken	EPI585420	2.3.2.1A
81	A/crow/India/01CA13/2012	Corvus	EPI837669	2.3.2.1A
82	A/crow/India/01CA16/2012	Corvus	EPI644444	2.3.2.1A
83	A/chicken/India/01CA09/2012	Chicken	EPI837644	2.3.2.1A
84	A/chicken/India/01CL1196/2012	Chicken	EPI644438	2.3.2.1A
85	A/yellow billed duck/Bhutan/358/2012	Anas undalata	EPI527311	2.3.2.1A
86	A/crow/India/02CA02/2012	Corvus	EPI642482	2.3.2.1A
87	A/crow/India/02CA03/2012	Corvus	EPI644417	2.3.2.1A
88	A/crow/India/02CA06/2012	Corvus	EPI644285	2.3.2.1A
89	A/crow/India/02CA07/2012	Corvus	EPI642487	2.3.2.1A
90	A/chicken/India/03CA02/2012	Chicken	EPI642472	2.3.2.1A
91	A/chicken/India/03CA03/2012	Chicken	EPI642475	2.3.2.1A
92	A/crow/India/03CA06/2012	Corvus	EPI644506	2.3.2.1A
93	A/chicken/India/04MO04/2012	Chicken	EPI623578	2.3.2.1A
94	A/chicken/India/04MO03/2012	Chicken	EPI623577	2.3.2.1A
95	A/turkey/India/10CA04/2012	Turkey	EPI644376	2.3.2.1A
96	A/duck/Bhutan/01CL03/2013	Duck	EPI644394	2.3.2.1A
97	A/duck/Bhutan/01TR03/2013	Duck	EPI644398	2.3.2.1A

98	A/northern pintail/India/03OR320/2013	Anas acuta	EPI644448	2.3.2.1A
99	A/chicken/India/03CA02/2013	Chicken	EPI623573	2.3.2.1A
100	A/chicken/India/07CA02/2013	Chicken	EPI558708	2.3.2.1A
101	A/chicken/India/08CA03/2013	Chicken	EPI558713	2.3.2.1A
102	A/chicken/India/12CL2168/2013	Chicken	EPI644407	2.3.2.1A
103	A/chicken/India/12CA01/2013	Chicken	EPI644404	2.3.2.1A
104	A/crow/India/01CA02/2014	Avian	EPI703578	2.3.2.1A
105	A/duck/India/11CA02/2014	Duck	EPI758769	2.3.2.1C
106	A/duck/India/11CA08/2014	Duck	EPI758761	2.3.2.1C
107	A/duck/India/11TR03/2014	Duck	EPI758753	2.3.2.1C
108	A/duck/India/12TR04/2014	Duck	EPI758777	2.3.2.1C
109	A/duck/India/12CA05/2014	Duck	EPI736906	2.3.2.1C
110	A/turkey/India/01CA01/2015	Turkey	EPI758745	2.3.2.1C
111	A/duck/India/03CA01/2015	Duck	EPI758785	2.3.2.1C
112	A/chicken/India/04CA17/2015	Chicken	EPI758793	2.3.2.1A
113	A/chicken/Bhutan/04TI01/2015	Chicken	EPI653111	2.3.2.1A
114	A/duck/India/10CA01/2016	Duck	EPI858836	2.3.4.4B
115	A/painted stork/India/10CA03/2016	Other avian	EPI858844	2.3.4.4B

current epidemiological needs. In conclusion, this modified assay provides a robust and flexible alternative for AI H5 diagnostics. Diagnostic laboratories may choose to use either the original or modified assay depending on the sequences of the circulating strains, offering flexibility without compromising accuracy.

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DECLARATIONS

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical standards: This article does not contain any studies with human or animal subjects.

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