



Development of a Novel Korean H9-Specific rRT-PCR Assay and Its Application for Avian Influenza Virus Surveillance in Korea

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Abstract

Since the 2000s, the Y439 lineage of H9N2 avian influenza virus (AIV) has been the predominant strain circulating in poultry in Korea; however, in 2020, the Y280 lineage emerged and spread rapidly nationwide, causing large economic losses. To prevent further spread and circulation of such viruses, rapid detection and diagnosis through active surveillance programs are crucial. Here, we developed a novel H9 rRT-PCR assay that can detect a broad range of H9Nx viruses in situations in which multiple lineages of H9 AIVs are co-circulating. We then evaluated its efficacy using a large number of clinical samples. The assay, named the Uni Kor-H9 assay, showed high sensitivity for Y280 lineage viruses, as well as for the Y439 lineage originating in Korean poultry and wild birds. In addition, the assay showed no cross-reactivity with other subtypes of AIV or other avian pathogens. Furthermore, the Uni Kor-H9 assay was more sensitive, and had higher detection rates, than reference H9 rRT-PCR methods when tested against a panel of domestically isolated H9 AIVs. In conclusion, the novel Uni Kor-H9 assay enables more rapid and efficient diagnosis than the “traditional” method of virus isolation followed by subtyping RT-PCR. Application of the new H9 rRT-PCR assay to AI active surveillance programs will help to control and manage Korean H9 AIVs more efficiently.

Keywords H9N2 · Low pathogenicity avian influenza virus · Real-time RT-PCR · Live bird market

Introduction

The first case of poultry infected with low pathogenicity avian influenza virus (LPAIV) H9N2 was officially reported on a turkey farm in Wisconsin, USA, in 1966 (Homme & Easterday, 1970). Since the mid-1990s, H9N2 avian influenza virus (AIV) has been identified worldwide, and the disease has not been eradicated in many countries in Asia and

the Middle East (Alexander, 2007; Davidson et al., 2014; Guo et al., 2000). H9N2 is a major subtype of AIV that has adverse effects on the global poultry industry (Alexander, 2007; Guo et al., 2000; Peacock et al., 2019). Although H9N2 AIV is classified as low pathogenicity, it causes mortality or egg drop depending on the breeding environment or co-infection with other pathogens, leading to severe economic losses (Guo et al., 2000; Lai et al., 2021; Lee et al., 2007; Mo et al., 2003). In general, H9N2 viruses are divided into North American and Eurasian types, and the Eurasian type is further classified into G1, Y280, and Y439 lineages based on the sequence of the hemagglutinin (HA) gene (Dong et al., 2011; Guo et al., 2000).

In Korea, the H9N2 AIV belonging to the Y439 lineage was first reported in poultry in 1996. Subsequently, it spread nationwide and became endemic in the 2000s; these viruses are now classified as the Korean lineage (Lee & Song, 2013; Lee et al., 2007; Mo et al., 2003, 2016). Vaccination against Y439 lineage H9N2 AIV was introduced in 2007, the main aim being to reduce economic losses to the layer and broiler breeder chicken industries. However,

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despite the H9N2 vaccination program, the virus continues to circulate, mainly within small-scale native Korean chicken farms and live bird markets (LBMs) (Choi et al., 2008; Lee et al., 2010; Youk et al., 2020). In June 2020 (and for the first time in Korea), the Y280 lineage of H9N2 AIV was isolated from chickens at an LBM (Youk et al., 2020). Since then, this lineage of H9N2 AIV has spread quickly nationwide, and is now the predominant strain among domestic poultry (Lai et al., 2021).

The AI national surveillance program in Korea relies on conventional real-time reverse transcription PCR (rRT-PCR) targeting the M, H5, or H7 genes, which enables rapid detection and diagnosis of high pathogenicity AIV (HPAIV). Although South Korea uses a subtyping RT-PCR assay and full-sequencing of the HA segment to identify the H9N2 virus, many other countries also employ various H9-specific rRT-PCR assays to identify the detailed distribution of various H9N2 lineages, including viruses of wild bird origin (El Khantour et al., 2020; Monne et al., 2008). Thus, occurrence of multiple H9N2 AIV lineages in South Korea means that we need to develop and evaluate the sensitivity and specificity of conventional H9 AIV-specific diagnostic assays that can detect multiple lineages of H9 viruses. To address this unmet need, we developed and evaluated a novel H9 AIV rRT-PCR assay (called the Uni Kor-H9 assay) based on highly specific universal primer/probe sets. This assay enables rapid, accurate, and efficient detection of the Y280 and Y439 lineages of Korean H9N2 AIVs.

Materials and Methods

LBM Surveillance and Virus Isolation

The nationwide LBM surveillance system in Korea is based on laboratory testing of clinical samples from live poultry on sale; these samples are collected from every registered poultry store two to four times a year depending on location. Briefly, clinical specimens are collected by the Livestock Health Control Association (LHCA) and sent to the Animal and Plant Quarantine Agency (APQA) or to the provincial veterinary service laboratories (VSLs), where they are tested for AIVs. Oropharyngeal (OP) swabs and cloacal (CL) swabs (or feces) are collected from the Korean native chickens, broiler chickens, and domestic ducks, which are the main species sold at LBMs. Detection of H9 AIV was confirmed by a subtyping RT-PCR assay after isolating the virus from embryonated eggs; positive samples (cycle threshold (Ct) \leq 40) were screened using the AIV M gene detection kit (Median Diagnostics, VR8100).

Primer and Probe Design

To differentiate the specific lineages of H9N2 viruses, several primers and probes were designed based on lineage-specific sequence information. Briefly, 67 complete HA gene sequences of H9 AIVs isolated in Korea were downloaded from the Korea Animal Health Integrated System (KAHIS) (<https://home.kahis.go.kr>) and the Global Initiative on Sharing All Influenza Database (GISAID) (<https://gisaid.org>): these sequences comprised 29 representative H9 AIVs belonging to the Y280 lineage, isolated since June 2020; 19 representative viruses selected from the Y439 lineage, isolated from 1996 to 2018; and 19 representative H9Nx AIVs, isolated from wild birds during 2020–2021. All sequences were edited and analyzed using the CLC Main Workbench program (version 6.9.1).

RNA Extraction and rRT-PCR Assay

Viral RNA was extracted from each reference strain and clinical isolate using the Maxwell RSC SimplyRNA Tissue Kit and the Maxwell RSC 48 instrument (Promega, AS1340). All H9 rRT-PCR analyses were performed using the CFX96 Touch Real-time PCR Detection System (C1000 Touch, Bio-Rad) and the QuantiNova Probe RT-PCR Kit (Qiagen, 208354). The volume of the reaction mixture for the H9 rRT-PCR assay was 20 μ l, comprising 2 \times QuantiNova Probe RT-PCR Master Mix (10 μ l), QN Probe RT-Mix (0.2 μ l), 20 \times primer-probe mix (0.8 μ M each forward and reverse primer and 0.2 μ M TaqMan Probe; 1 μ l of each), template RNA (5 μ l; \leq 400 ng/reaction), and 3.8 μ l of RNase-Free Water. The H9 rRT-PCR conditions were as follows: 45 $^{\circ}$ C for 10 min, followed by 95 $^{\circ}$ C for 5 min, and then 40 cycles of 5 sec at 95 $^{\circ}$ C and 30 sec at 60 $^{\circ}$ C. The amplification data were collected and analyzed using Bio-Rad CFX Maestro 1.1 software (Version: 4.1.2433.1219).

Analytical Specificity

The specificity of the novel H9 rRT-PCR assay was tested using each reference H1–H15 subtype virus. In addition, other major avian viruses such as Newcastle disease virus (NDV), Infectious bursal disease virus (IBDV), Infectious Bronchitis virus (IBV), Fowl Pox virus (FPV), and Chicken infectious anemia virus (CIAV), as well as major bacterial pathogens, were tested. Furthermore, 18 AIV-negative specimens were used as controls. The complete list of samples tested for analytical specificity is provided in the Supplementary data 3.

Analytical Sensitivity

The sensitivity of the novel H9 rRT-PCR assay was evaluated using viruses representative of the Y280 and Y439 lineages, which were isolated from poultry and wild birds. The full-size HA genes of each representative virus were amplified using the primers and method proposed by Hoffmann et al. (2001). The amplicon was then confirmed and purified using the QIAquick Gel Extraction Kit (Qiagen). The concentration of the final purified product was measured using a NanoDrop One Spectrophotometer (ThermoFisher Scientific). Finally, the copy number of the full-size HA gene was quantified using the method described by CEL-seq (<http://cels.uri.edu/gsc/cndna.html>). These copy numbers were serially diluted ten-fold to obtain a “standard” sample for each concentration. To evaluate the standard rRT-PCR assay, each sample was tested three times.

Comparison with Other H9 rRT-PCR Assays

The sensitivity of the novel H9 rRT-PCR assay for the H9 subtype was compared with that of other diagnostic methods reported by IZSve (Istituto Zooprofilattico Sperimentale delle Venezie, Italy) and NIID (National Institute of Infectious Diseases, Japan) (Monne et al., 2008; Saito et al., 2019). The rRT-PCR assay conditions and the primer/probe sets used have been reported previously. Fifty-six viruses isolated from 2016 to 2021 were used, all belonging to the Y439 and Y280 lineages isolated from poultry in LBMs or from wild birds.

Agreement Between the New H9 rRT-PCR Assay and Virus Isolation Method

Clinical samples obtained from LBMs were used to evaluate the correlation between the sensitivity of the virus isolation method and the rRT-PCR assay. Briefly, 301 clinical samples, including OP and CL swabs and feces, collected from Korean native chickens in 2020–21 were used, and the diagnostic results were compared. Of these, 142 samples (118 OP swabs and 24 feces samples) identified as AIV M gene-positive were selected and tested using the two methods: virus isolation after egg inoculation, and the new H9 rRT-PCR assay. The virus isolation results were confirmed by a hemagglutination test, and the HA subtype of the isolates was determined using the LiliF AIV Multi-tube RT-PCR Kit (iNtRON Biotechnology, IPC11009).

Analytical Reproducibility

To evaluate the performance of the novel H9 rRT-PCR assay in a field setting, 29 clinical samples (including 13 AIV genome-positive swabs) were shared and tested

independently at the APQA and two VSLs (Gyeonggi and Gyeongsangbuk-do). The results were expressed as a correlation coefficient (CC), as described previously (Mirzaei et al., 2018).

Statistical Analysis

Data were analyzed using Prism version 5.0 software (GraphPad Software). Comparison of Ct values between groups was made by one-way analysis of variance (ANOVA). The threshold of Ct value was analyzed using the log-rank test. $P < 0.05$ was considered statistically significant.

Results

Circulation of Multiple Lineages of H9 AIVs in Korea

The Y439 lineage of Korean H9 AIV was detected in poultry in South Korea from 1996 to 2018. Although the Y439 lineage of H9N2 AIV has not been detected in domesticated poultry since 2018, the total number of H9N2 detections in Korea has increased due to emergence of the Y280 lineage of H9 AIV in LBMs in 2020. Since then, the Y280 lineage has spread gradually to domestic poultry farms, including layer chickens and Korean native chickens, and become the dominant H9 AIV strain nationwide (Fig. 1). In addition, the Y439 lineage of wild bird-origin (Eurasian lineage) H9 AIVs (Han et al., 2019; Suttie et al., 2019) has been detected continuously during annual wild bird surveillance; this virus is another potential source of infection of poultry (Supplementary data 1).

Design and Evaluation of Universal Primer and Probe Set for H9 AIV

To design the universal primer and probe specific for H9N2 AIVs, we first constructed phylogenetic trees based on the complete HA gene sequences of H9Nx AIVs isolated from Korea and other countries (these sequences were obtained from the KAHIS and GISAID). As shown in Fig. S1, the H9 subtype AIVs were classified genetically into three groups: Y280, Korean Y439, and wild bird-origin Eurasian Y439 H9 lineages. Based on sequence analysis, we designed a new diagnostic assay (the Uni Kor-H9 assay) that can detect Y280, Korean Y439, and wild bird-origin Eurasian Y439 H9 lineages simultaneously (Table 1).

To evaluate the specificity of the new Uni Kor-H9 assay, we tested different subtypes of AIV ($n = 14$), other avian viruses ($n = 5$), and bacteria found in poultry ($n = 18$) (see Supplementary data 3). As expected, all H9N2 viruses generated a positive signal for the M and H9 genes, regardless of lineage. However, all AIV subtypes other than H9 showed

Fig. 1 Number of positive H9 AIV cases detected in South Korea from 2017 to 2022. H9 AIVs were detected in domestic poultry, at LBMs, and in wild birds through an AI surveillance program. These results show detection rates of H9 from 2017 to 2022 (H9 AIV lineages and origins are shown)

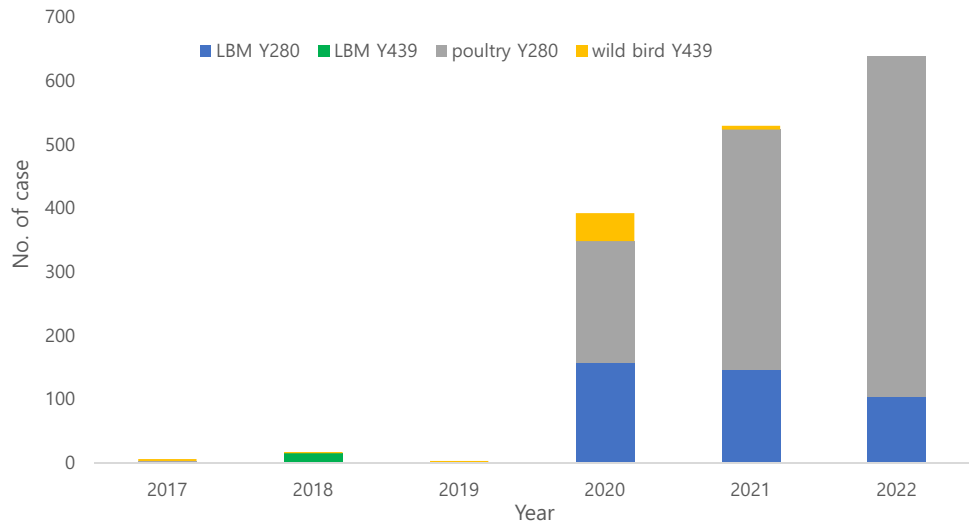


Table 1 Sequences of the primers and probes used for the H9 rRT-PCR assay

Name	Nucleotide sequence (5'–3')	Position	Size (bp)
Uni Kor-H9-F	CAGAAAATAGARGGGGTMAARCTGG	1525–1549	108
Uni Kor-H9-R	RAAGGCAGCAAACCCYATTGC	1612–1632	
Uni Kor-H9-Probe	FAM-GACTGTGCGCCTCATCTCTGTG-BHQ1	1587–1608	

a positive signal only for the M gene (Ct value 15.38 ± 1.4). Furthermore, the new assay did not show any cross-reactivity with other major avian respiratory bacterial or viruses (i.e., NDV, IB, IBV, and CIAV; Table 2).

The analytical sensitivity of the Uni Kor-H9 assay was determined by measuring the limit of detection using H9N2 viruses that are representative of each lineage (i.e., A/chicken/Korea/LBM261/2020 [Y280], A/chicken/Korea/LBM294/2018 [Y439], A/chicken/Korea/01310/2001 [Vaccine strain of Y439], and A/wild bird/Korea/H276/2021 [Eurasian Y439]). Quantitative rRT-PCR analysis demonstrated that the R^2 values from standard curve analyses ranged from 0.993 to 0.998 (Table 3). The virus belonging to the Y280 lineage, A/chicken/Korea/LBM261/2020, was detected at a copy number as low as 7.3, while the A/chicken/Korea/LBM294/2018, A/chicken/Korea/01310/2001 and A/wild bird/Korea/H276/2021 were detected at copy numbers of 478.5, 88.04 and 19.93, respectively (Table 3).

Comparison with Other H9 rRT-PCR Assays

To evaluate the efficacy of the Uni Kor-H9 assay, we tested a panel of viruses using different rRT-PCR methods. A comparison of the relative sensitivity of these H9 rRT-PCR assays revealed that the Uni Kor-H9 assay had the highest sensitivity for all three lineages: Y280, Korean Y439, and wild bird-origin Eurasian Y439 viruses (Fig. 2 and Supplementary data 2). The Uni Kor-H9 assay detected all 56

viruses, whereas the NIID and IZSve methods detected only 47 and 41 viruses, respectively. In addition, the deviation of the detection values per virus group for these says was greater than that of the Uni Kor-H9 assay (based on a positive Ct value of ≤ 40). The sensitivity of the NIID method (Saito et al., 2019) for the Y280 lineage of H9 AIV was comparable with that of the Uni Kor-H9 assay, whereas the sensitivity of the NIID for both Y439 lineages in poultry and wild birds was quite low. The IZSve method (Monne et al., 2008) showed relatively low sensitivity for all three lineages.

The reproducibility of the Uni Kor-H9 assay was further tested at the APQA and two VSLs (Gyeonggi and Gyeong-sangbuk-do). Briefly, 29 known samples (13 positive and 16 negative) were tested in the Uni Kor-H9 assay, and all three laboratories obtained the same results with respect to positivity or negativity. The standard deviation of the Ct value for positive samples among laboratories ranged from 0.78 to 2.21 (Table 4). The CC for pairwise comparison of positive samples was 0.87–0.93 (Table 5).

Agreement Between the H9 rRT-PCR Assay and the Virus Isolation Method

Finally, 142 clinical specimens (118 OP swabs and 24 feces specimens, all of which tested positive using the M rRT-PCR detection kit) were tested using the Uni Kor-H9 assay to evaluate its efficacy. Of these, 136 tested positive in the H9 rRT-PCR, whereas only 76 specimens (66 OP

Table 2 Specificity of the rRT-PCR assay for other AIV subtypes, avian viruses, and avian bacteria

Subtype	Ct value in rRT-PCR assay (average of Ct value ± standard deviation)	
	M gene	H9 gene
H1	12.74 ± 1.67	ND
H2	12.83 ± 0.73	ND
H3	13.93 ± 0.71	ND
H4	13.93 ± 0.37	ND
H5	14.23 ± 1.13	ND
H6	13.12 ± 1.91	ND
H7	13.07 ± 1.11	ND
H8	13.38 ± 0.30	ND
H10	12.42 ± 1.10	ND
H11	14.53 ± 0.43	ND
H12	13.40 ± 0.10	ND
H13	16.43 ± 1.0	ND
H14	14.69 ± 0.46	ND
H15	14.11 ± 1.13	ND
H9 (Y439)	18.08 ± 1.24	19.33 ± 2.64
H9 (Y280)	11.13 ± 0.95	10.54 ± 1.07
H9 (EU WB) ^a	17.44 ± 1.21	20.66 ± 0.61
H9 (clinical) ^b	20.28 ± 0.78	22.79 ± 1.30
Other avian viruses ^c	ND	ND
18 bacteria ^d	ND	ND

ND not detected

^aEurasian Wild birds

^bClinical samples including swab and feces collected from birds

^cViruses(excluding AIV) isolated from birds

^dBacteria isolated from birds

swabs and 10 feces) tested positive in the virus isolation assay (Supplementary data 4). When compared with the results obtained using the conventional AIV M gene detection kit, the sensitivity of the Uni Kor-H9 assay was 95.8% (based on 100% positivity of 142 samples), which is much higher than that of the virus isolation method (53.5%).

Table 3 Sensitivity and limit of detection of the Uni Kor-H9 assay for representative viruses belonging to the Y439 and Y280 lineages

Virus (lineage)	Copy number of viral stock (copies/μl)	PCR efficiency (%)	R ²	Limit of detection ^a (copy number)
A/chicken/Korea/LBM261/2020 (Y280)	7.319 × 10 ⁹	106.9	0.995	7.319
A/chicken/Korea/01310/2001 (Y439)	8.804 × 10 ⁹	95.9	0.993	88.04
A/chicken/Korea/LBM294/2018 (Y439)	4.785 × 10 ⁹	120.7	0.993	478.5
A/wild bird/Korea/H276/2021 (Y439)	1.993 × 10 ⁸	114.7	0.998	19.93

^aMean of three repeat measurements

Discussion

Since 2008, a large-scale AI active surveillance program has been implemented in South Korea for early detection of HPAI viruses (Mo et al., 2016; Sagong et al., 2023). To this end, wild birds (feces, captured wild birds, and carcasses), domestic poultry (chickens, domestic ducks, and other poultry), LBMs, and poultry traders have been targeted for periodic testing by rRT-PCR of the viral M, H5, and H7 genes. This national AI active surveillance program has detected high pathogenicity H5Nx AIVs in wild birds at the early stages of their migration, before outbreaks occur in poultry (Baek et al., 2021; Sagong et al., 2022). The program also detected the Y280 lineage of H9N2 AIV in LBMs (Choi et al., 2005; Heo et al., 2021; Liu et al., 2003). The Y280 lineage of H9N2 AIV began circulating in LBMs in China and Southeast Asia, from where it spread to neighboring regions and countries (Chen et al., 2016, 2017; Lin et al., 2017; Nomura et al., 2012; Novianti et al., 2019; Suttie et al., 2019). The genetic diversity of H9N2 AIV increases during circulation in LBMs, which may increase the chances of viral transmission to other species, including humans (Chen et al., 2015; Lee et al., 2010; Li et al., 2014). Therefore, continuous and comprehensive monitoring of poultry farms and LBMs, as well as genetic characterization of H9 AIVs, is essential for early detection of AIVs and for raising the alarm regarding public health concerns. To achieve this, a more accurate, simple, and sensitive diagnostic assay is required.

The standard method for H9 subtyping for AI surveillance in Korea involves a combination of virus isolation and RT-PCR; however, this is both time-consuming and labor-intensive. The rRT-PCR assay used to detect the M, H5, and H7 genes has been used for AI surveillance; however, the H9 rRT-PCR assay was not introduced until recently (Kim et al., 2013).

Here, we developed a new H9 rRT-PCR assay that was more sensitive, specific, and reproducible than current detection methods, including the virus isolation method. The new assay detected both Y439 and Y280 lineage AIVs in clinical specimens obtained from poultry and wild birds. There were

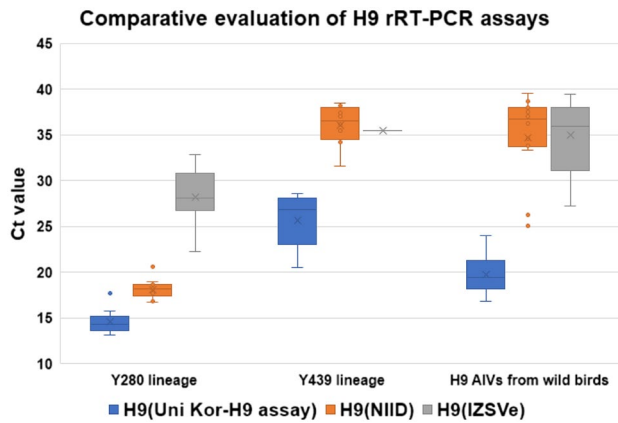


Fig. 2 Comparison of the relative sensitivity of the H9 rRT-PCR assay with that of two different rRT-PCR methods. Fifty-six H9N2 viruses were tested, including 26 belonging to the Y280 lineage, 15 belonging to the Y439 lineage isolated from poultry, and 15 belonging to the Y439 lineage isolated from wild birds. Blue bars indicate the Ct value for the Uni Kor-H9 assay, orange bars indicate the Ct value for the H9 rRT-PCR assay developed by NIID (Saito et al., 2019), and gray bars indicate the Ct value for the H9 rRT-PCR assay developed by IZSve (Monne et al., 2008)

Table 4 Reproducibility of the Uni Kor-H9 assay conducted in three different laboratories

Sample no. ^a	H9 Ct value (inter-assay)			Mean ± SD
	D1 ^b	D2 ^b	D3 ^b	
1	36.43	35.40	38.05	36.61 ± 1.31
2	28.53	27.40	31.21	28.98 ± 1.84
3	33.05	30.30	32.80	32.05 ± 1.52
4	31.30	27.80	31.30	30.13 ± 2.02
5	30.39	26.30	29.82	28.83 ± 2.21
6	32.72	30.10	33.49	32.11 ± 1.78
7	34.50	33.13	35.08	34.20 ± 1.08
8	34.25	32.46	33.85	33.38 ± 1.21
9	31.91	29.90	33.21	31.67 ± 1.66
10	33.74	32.50	36.04	34.08 ± 1.77
11	31.38	31.56	32.53	31.63 ± 0.78
12	31.61	30.50	32.31	31.47 ± 0.91
13	31.35	29.20	32.46	31.02 ± 1.68
14–29	ND	ND	ND	–

ND not detected

^a1–13 were H9-positive clinical samples; 14–29 were H9-negative samples

^bD1, D2, and D3 indicate the lab at APQA and the two different VSL (Gyeonggi and Gyeongsangbuk-do), respectively

no false positives or cross-reactions with other pathogens, including other AIV subtypes, avian viruses, and avian bacteria (Table 2). Furthermore, the diagnostic sensitivity of the Uni Kor-H9 assay kit (i.e., a relative sensitivity of 95.8%

Table 5 Correlation coefficients for the three different laboratories conducting the Uni Kor-H9 assay

	D1	D2	D3
D1		0.89	0.87
D2	0.89		0.93
D3	0.87	0.93	

Table 6 Agreement between the Uni Kor-H9 assay and the virus isolation method

Result	Type of sample				Total
	OP ^a		Feces		
	VI+ ^b	VI- ^c	VI+	VI-	
H9+ (M+) ^d	66	49	10	11	136
H9- (M+) ^e	NT ^f	3	NT	3	6
Total	66	52	10	14	142

The Uni Kor-H9 assay was compared with the virus isolation method (egg inoculation)

^aOropharyngeal swab

^bVirus Isolation-positive

^cVirus Isolation-negative

^dPositive for the H9 and M genes by rRT-PCR

^eNegative for the H9 gene and positive for the M gene by rRT-PCR

^fNot tested

for clinical samples collected for LBM surveillance) was comparable with that of a commercial AIV M gene detection assay (Table 6). In addition, when using selected samples with detectable M genes, we found that detection rate of the Uni Kor-H9 assay was higher than that of the conventional diagnostic method (i.e., virus isolation) (Mirzaei et al., 2018). However, the Uni Kor-H9 assay also yielded false negative results for clinical samples containing very low concentrations (10^0 to 10^1 EID₅₀/ml) of virus (data not shown).

Although the analytical sensitivity of the Uni Kor-H9 assay for Y439 lineage viruses appeared to be lower than that for the Y280 lineage, the assay demonstrated a broad detection range of H9 lineage, and showed particularly high sensitivity for the Y280 lineage, which has recently become dominant in Korea (Fig. 1 and Table 3). In contrast to representative viruses from other lineages that did not exhibit any mismatches in the primer/probe sequences, the two representative H9 AIVs belonging to the Y439 lineage showed a single mismatch at the reverse primer binding site. This mismatch may have contributed to the lower limit of detection for the Y439 lineage (copy number, 88.04–478.5).

To verify the efficacy of the Uni Kor-H9 assay for field application, it was tested in two field surveillance studies conducted during the 2022 season, which showed it to be a useful method of AI surveillance. Furthermore, the Uni Kor-H9 assay was more efficient than previously reported

diagnostic methods used to identify H9N2 viruses, including recent H9 AIVs detected in poultry or wild birds in Korea (Fig. 2). Although our new H9 rRT-PCR assay may need further evaluation in large-scale studies, and with a single set of primers and probes, to determine whether it can identify all variations or mutations occurring during continuous virus circulation (especially of H9 AIVs) (Slomka et al., 2013), the results clearly demonstrate that the assay is more suitable than other methods for monitoring H9 AIVs circulating recently in Korea.

To summarize, we developed a highly efficient rRT-PCR assay that detects a broad range of H9 AIVs, including Y280 and various Y439 lineages, and evaluated its efficacy by comparing it with currently used methods. Furthermore, we used the assay to test a large number of clinical specimens from AI surveillance programs, and found that it was as sensitive and specific as commercial AIV M diagnostic kits, and more sensitive and specific than the virus isolation method. These results suggest that this new assay can be used to detect and identify Korean H9 AIV quickly and efficiently, making it an effective replacement for existing diagnostic methods. Although it may need continuous development and updates, we believe that this assay is suitable for large-scale surveillance of a broad range of H9 AIVs present on domestic poultry farms and in wild bird habitats.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12275-023-00088-8>.

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Data Availability GISAID: <https://platform.epicov.org/epi3/cfrontend>.

Declarations

Conflict of Interest The authors declare no conflicts of interest.

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