

# Development and evaluation of a one-step real-time RT-PCR assay for universal detection of influenza A viruses from avian and mammal species

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**Abstract** The objective of our study was to develop and evaluate a TaqMan real-time RT-PCR (RRT-PCR) assay for universal detection of influenza A (IA) viruses. The primers and LNA-modified octanucleotide probe were selected to correspond to extremely conserved regions of the membrane protein (MP) segment identified by a comprehensive bioinformatics analysis including 10,405 IA viruses MP sequences, i.e., all of the sequences of the Influenza Virus Sequence database collected as of August 20, 2009. The RRT-PCR has a detection limit of approximately five copies of target RNA/reaction and excellent

reaction parameters tested in four IA viruses reference laboratories. The inclusivity of the assay was estimated at both the bioinformatic and the experimental level. Our results predicted that this RRT-PCR assay was able to detect 99.5% of known human IA virus strains, 99.84% of pandemic influenza A (H1N1) strains, 99.75% of avian strains, 98.89% of swine strains, 98.15% of equine strains, and 100% of influenza A viruses of other origin.

## Introduction

Influenza A (IA) virus is one of the most important human and veterinary pathogens. Various IA virus subtypes are causative agents of influenza, which is a significant zoonosis with high morbidity or mortality in different avian and mammal species worldwide involving humans [2, 14].

The IA virus genome consists of eight negative-sense RNA segments. The intrinsic genetic instability of the virus results from its high mutability and genetic reassortment, combined with interspecies transmission, establishment of host-specific lineages and geographical separation of host species has led to a considerable genetic and antigenic heterogeneity and evolutionary diversity. Therefore, the development of universal methods to detect as many genetic variants of the virus as possible is of crucial importance for IA virus surveillance, especially in avian species, which are considered the natural reservoir of IA virus [11, 14, 19].

Currently, conventional, or more recently, real-time RT-PCR (RRT-PCR) represents the leading technique in IA virus diagnosis, implemented as the first line of screening in many laboratories. Conventionally, these RT-PCR screening methods were designed to detect IA virus on a universal basis by amplifying a selected portion of the

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membrane protein (MP) gene segment [3–5, 9, 11, 16, 18, 21]. The MP gene segment is a bicistronic RNA molecule encoding two proteins, M1 and M2. The gene that encodes the M1 protein is considered to be highly conserved across various IA virus subtypes and host species. Nevertheless, phylogenetic analysis of a large panel of MP sequences has revealed that the sequence conservation is at the amino acid rather than the nucleotide sequence level, where the latter exhibits significant variation in both the M1 and M2 genes [8, 20]. Accordingly, mutations in the MP gene segment result predominantly in synonymous codon changes, mostly in the M1 gene [8, 17, 20], which constitutes 73.6% of the whole segment.

Although synonymous codon variation apparently plays a minor evolutionary role, it is of crucial importance in molecular diagnosis of IA viruses. The nucleotide mutations falling within the primer- and probe-binding regions may result in false negativity due to amplification or signal detection failure.

To date, several universal RT-PCR and RRT-PCR techniques for the detection and monitoring of IA viruses have been developed [3–5, 9, 11, 16, 18, 21]. Although all of these techniques follow a common strategy for universal assay design, i.e. selection of primers and probes on the basis of the most conserved sequences within the MP segment, the sequence complementarity within the primer- and probe-binding regions is often not confirmed by comparison of a large set of MP sequences, possibly because only a small number of sequences were available at that time. Therefore, it remains unclear how many IA virus strains go undetected and what inclusivity can be expected when a given assay is employed.

The aim of this work was to develop an RRT-PCR assay for the universal detection of IA viruses from all known host species and to determine the inclusivity of the assay both on the theoretical and the experimental level. The assay was extensively validated for all diagnostic test criteria through the joint efforts of four IA virus reference laboratories.

## Materials and methods

### Influenza A viruses

A collection of 320 IA virus strains representing 42 different subtype combinations was analysed. The samples included alantoic fluids, lyophilized cell cultures, and IA-virus-positive field specimens (organ suspensions, cloacal, tracheal, nasal and nasopharyngeal swabs and bronchoalveolar lavages). The IA virus panel contained 69 human, 30 pandemic influenza A (H1N1), 177 avian, 26 swine and 18 equine strains representing 15 hemagglutinin and 9

neuraminidase subtypes, i.e. 42 subtype combinations (Supplementary Table S1). The strains originated in both Eurasia and North America and encompassed a collection period from 1947 to 2009. All specimens were selected from the repositories of the State Veterinary Institute Prague (SVIP) and National Institute of Public Health (NIPH), Czech Republic, the State Veterinary Institute Zvolen (SVIZ), Slovak Republic, and the Central Agricultural Office (CAO), Hungary.

### Field samples

Field samples used in surveillance included 3,323 pooled organ suspensions and cloacal or tracheal swabs collected from birds and 53 equine nasal swabs. The samples were collected during the surveillance of avian IA viruses in the Czech Republic in 2007–2009. The birds were of 29 avian species including both wild and domestic birds.

### Nucleic acid extraction

RNA or total nucleic acid was extracted using a MagNA-Pure Compact, MagNAPure LC (both from Roche), Biorobot M48 (Qiagen), X-tractor Gene (Corbett Robotics) extractors or an RTP<sup>®</sup> DNA/RNA Virus Mini Kit (Invitex). The MagNAPure instruments used the Total Nucleic Acid Extraction Kit with input sample volumes of 200 or 400  $\mu$ l and elution volumes of 50 or 100  $\mu$ l. Extraction with the Biorobot M48 was performed using a MagAttract Viral RNA M48 Kit with input and elution volumes of 300 and 50  $\mu$ l, respectively. The X-tractor Gene device employed the SV96 Total RNA Isolation System (Promega) with input and elution volumes of 100 and 50  $\mu$ l, respectively.

### Selection of RRT-PCR primers and probe

The PCR primers were selected as a result of comprehensive bioinformatics analysis. To identify conserved regions within the IA virus MP segment, all of the MP sequence data in the Influenza Virus Sequence Database (ISD; [1]) were analysed, representing 8,747 sequences (reference date April 20, 2009). The sequences were aligned separately for each main host: avian, human, swine and equine. The remaining sequences were joined into one data pool called “others”. Multiple sequence alignments were constructed on the basis of 4,125 human, 3,896 avian, 484 swine, 96 equine and 146 other sequences. Each sequence pool was aligned using the MAFFT program (Multiple Alignment using Fast Fourier Transformation) [10] employing the FFT-NS-1 or FFT-NS-2 method. Subsequently, alignment editing and positional nucleotide numerical summary calculation was performed using the BIOEDIT software [6].

In order to express the degree of nucleotide variation at each position of the MP segment, the amount of variability through a column in the alignment was calculated as informational entropy,  $H(x)$ , defined by Shannon:

$$H(x) = - \sum_{i=1}^x f(i, x) \ln f(i, x)$$

where  $f(i, x)$  is the frequency of each base  $i$  at any position  $x$  in the multiple sequence alignment. Accordingly, the most conserved positions are those with the lowest entropy value (the highest informational content) converging on or equal to zero. The informational entropy was calculated separately for each sequence pool and visualised graphically as entropy plots. To reveal the overall nucleotide variation of the MP segment regardless the sequence origin, the partial entropy plots were assembled and visualised as the main entropy plot.

Based on extensive bioinformatic analysis, the primers and probe were designed based on regions with extremely low  $H(x)$  values, almost equal to zero. The forward primer SVIP-MP-F (5'-GGCCCCCTCAAAGCCGA-3') was selected from positions 77 to 93 and the reverse primer SVIP-MP-R (5'-CGTCTACGYTGCAGTCC-3'), which contained one degenerated position, was chosen from positions 242 to 258. The probe SVIP-MP-UPL104 represented an LNA (locked nucleic acid), modified and dual-labelled octanucleotide selected from the 165 Universal Probe Library, probe number 104 (Roche; the rights to the probe sequence are protected). The 5' end of the probe was labelled with FAM (fluorescein), and the 3' end with a dark quencher dye.

#### SVIP-MP RRT-PCR assay

A TaqMan quantitative real-time RT-PCR assay called SVIP-MP, which amplified a 182-nucleotide-long region of the IA virus MP segment (position 77–258) was developed. The SVIP-MP assay was designed as a one-step procedure using samples prepared with a OneStep RT-PCR kit or a QuantiTect Probe RT-PCR kit (both from Qiagen) in a final volume of 25  $\mu$ l containing 0.6  $\mu$ M of each primer, 0.2  $\mu$ M of a probe and 5  $\mu$ l of sample. For the OneStep RT-PCR kit, the concentration of  $MgCl_2$  was adjusted to 4 mM. The following thermoprofile was used: reverse transcription for 30 min at 50°C and 15 min at 95°C, followed by 45 cycles of 10 s at 95°C, 20 s at 60°C and 10 s at 72°C, with signal acquisition at the end of the 60°C period. The 60–72°C ramp temperature was decreased to 1°C/s. The assay was evaluated and optimised on a Light Cycler 480 instrument (Roche) using the Simple Probe detection format. In addition, the assay was examined on several additional real-time platforms: MJ MiniOpticon and iCycler (both

from BioRad), Rotor-Gene 2000 and 6000 (Corbett Robotics), Stratagene Mx30005P (Stratagene), Light-Cycler1.2 (Roche) and ABI7000 and ABI7500 (Applied Biosystems). For the Stratagene Mx30005P and ABI platforms, the 60°C period was elongated to 30 s. When possible, the 60–72°C temperature ramp was decreased to 1°C/s, or as slow as possible.

#### Preparation of RNA standard for RRT-PCR

An RNA standard was prepared from the A/duck/Czech Republic/14516/07 (H3N8) strain (further abbreviated as CZE/H3N8). The entire MP segment of CZE/H3N8 was amplified with T7-M-F: Bm-M-1027-R primer combinations. The primer T7-M-F was modified from the Bm-M-F primer [7] by replacing the first 14-base-long nucleotide tag with a T7 RNA polymerase promoter sequence: TA-ATACGACTCACTATAGGGCGAA. The reaction was performed using the OneStep RT-PCR kit (Qiagen) in a final volume of 25  $\mu$ l with 5  $\mu$ l of total nucleic acid extract. The cycling conditions were as described by Hoffmann et al. [7], with the elongation time shortened to 3 min. The amplicon was verified by sequencing.

In the next step, approximately 1  $\mu$ g of the CZE/H3N8 MP segment PCR product was transcribed in vitro using the RiboMAX Large Scale RNA Production System with T7 RNA polymerase (Promega) in a final volume of 100  $\mu$ l. The DNA template was removed by RQ1 DNase treatment, and the transcription mix was diluted to 200  $\mu$ l and extracted into a final volume of 100  $\mu$ l (MagNAPure Compact, total nucleic acid extraction kit). The amount of RNA template was determined by limiting dilution assay and Poisson analysis [12]. According to this approach, a diluted extract was subjected to amplification in replicates, and the average copy number of target molecules per unit volume was calculated as:

$$P_N = -\ln N_0/N_T$$

where  $N_0$  is the number of negative results and  $N_T$  is the total number of tests. The Poisson analysis indicated an average of 1.1 RNA copies/5  $\mu$ l in the final dilution of the standard. For easier expression, this value was rounded to 1 copy/5  $\mu$ l and was used for backward quantification of the standard stock.

#### Evaluation of the primers and probe and estimation of the inclusivity of the assay

The inclusivity of the assay was tested by two approaches.

The bioinformatic approach was based on a portion of the MP sequences in each particular alignment that had 100% complementarity to the primers and the probe. To this end, a re-evaluation of the sequence stability within the primer and

**Table 1** Influenza A virus MP gene sequences aligned for estimating the inclusivity of the SVIP-MP RRT-PCR assay

Species	No. of aligned sequences
Human	4,627
New human H1N1	694
Avian	4,249
Swine	546
Horse	119
Others	
Blow fly	1
Camel	1
Cat	3
Civet	1
Dog	7
Environment	116
Ferret	1
Leopard	1
Mink	2
Muskrat	1
Pika	5
Plateau pika	1
Raccoon dog	2
Seal	1
Stone marten	1
Tiger	5
Unknown	21
Total	10,405

probe binding regions was performed due to a rapid increase in the number of ISD MP sequences, especially regarding the emergence of the pandemic influenza A of H1N1 subtype in human population since April 2009. The re-evaluation was performed by aligning 10,405 MP sequences (downloaded on August 20, 2009) including 4,627 human, 694 pandemic influenza A (H1N1), 4,249 avian, 546 swine, 119 equine and 170 other MP sequences (Table 1).

**Fig. 1** Mutations within the forward (SVIP-MP-F) and reverse (SVIP-MP-R) primers (designated as FX1-16 and RX1-14) mimicking the original mutations within the primer-binding regions of the IA virus MP segment

	5	10	15		5	10	15
<b>SVIP-MP-F</b>	GGCCCC	TCAAAG	C	<b>SVIP-MP-R</b>	CGTCT	ACGYTG	CAGTCC
FX1	.....	.....	T..	RX1	.....	.....	C..
FX2	.....	..C..	AG...	RX2	.....	.....	G..
FX3	.....	..CT..	.....	RX3	.....	.....	T..
FX4	.....	.....	G..	RX4	.....	.....	C..
FX5	.....	..A..	.....	RX5	.....	..A..	.....
FX6	.....	.....	A..	RX6	.....	..C..	.....
FX7	.....	.....	AG...	RX7	.....	.....	T..
FX8	.....	.....	C..	RX8	.....	.....	T..
FX9	.....	..C..	.....	RX9	.....	.....	A..
FX10	.....	.....	TT..	RX10	.....	..A..	.....
FX11	.....	.....	T..	RX11	A..	A..	.....
FX12	.....	..C..	.....	RX12	A..	G..	.....
FX13	.....	.....	A..	RX13	.....	..G..	.....
FX14	.....	.....	T..	RX14	.....	..G..	.....
FX15	..A..	T..	.....				
FX16	.....	..A..	.....				

The experimental approach verified the ability of the SVIP-MP assay to tolerate mutations in the primer sequences. Sixteen mutated variants of the forward primer SVIP-MP-FX1:FX16 and 14 of the reverse primer SVIP-MP-RX: RX16 were designed that mimicked naturally occurring mutations and were considered as the most critical for primer binding (Fig. 1). Consequently, reactions were set up with each particular mutated SVIP-MP-FX or RX primer coupled with its original SVIP-MP-R or SVIP-MP-F counterpart and used to amplify the CZE/H3N8 RNA standard at three dilutions,  $10^2$ ,  $10^4$  and  $10^6$  copies/reaction, on the LightCycler 480.

#### Sensitivity of the assay

To determine the analytical sensitivity of the assay, a serial dilution of quantified CZE/H3N8 RNA standard (from  $10^7$  to  $10^1$  copies/reaction) was prepared. The standard curve constructed by plotting the cycle threshold values ( $C_T$ ) against the log of the standard dilution was obtained in two independent runs. Each run was performed in four replicates.

#### Specificity of the assay

To exclude cross-reactivity with other virus and bacterial species, a panel of 108 isolates representing a total of 24 human, avian, swine and equine viruses and a group of 27 bacteria was tested. The viruses included: influenza B and C viruses, human respiratory syncytial virus, human adenovirus, human herpes virus, avian infectious bronchitis virus, avian paramyxovirus 1-4, 6-9, pigeon paramyxovirus, psittacine beak and feather disease virus, porcine reproductive and respiratory syndrome virus, porcine circovirus 2, porcine herpes virus 1, porcine parvovirus, porcine coronavirus, classical swine fever virus, equine arteritis virus and equine herpes virus 1. The bacterial cultures included *Actinobacillus equuli*, *salpingitidis* and

*pleuropneumoniae*, *Bordetella bronchiseptica*, *Campylobacter jejuni*, *coli* and *lari*, *Enterococcus faecalis*, *Erysipelothrix rhusiopathiae*, *Escherichia coli*, *Candida rugosa*, *Chlamydia sp.*, *Gallibacterium anatis*, *Klebsiella pneumoniae*, *Mycobacterium tuberculosis* and *avium* complex, *Nicoletella semolina*, *Ornithobacterium rhinotracheale*, *Pasteurella mairii* and *multocida*, *Pseudomonas aeruginosa*, *Salmonella serovar Newport*, *Staphylococcus hyicus* and *aureus*, *Streptococcus suis*, *Taylorella equigenitalis* and *Volucribacter amazonae*. The list of virus and bacterial isolates is shown in Supplementary Table S2.

To exclude background hybridization, i. e. interference with avian and mammal genomic nucleic acids, and non-specific signal generation, a total of 42 IA-virus-negative specimens were tested, representing whole blood of humans and whole blood or pooled organ suspensions of swine, horse, seal, dog, cat, tiger, stone marten and of various bird species origin, including chicken, duck, goose, turkey, swan and gull.

### Reproducibility of the assay

The intra- and inter-assay reproducibility was determined for three dilutions of the CZE/H3N8 RNA standard:  $10^6$ ,  $10^4$  and  $10^2$  copies/reaction. The intra-assay variation was analysed in five replicates, and the inter-assay reproducibility was assessed in five independent runs. The statistical dispersion was expressed as standard deviation (SD) and the repeatability as relative standard deviation (RSD %) within and between the experiments.

### Comparison of the SVIP-MP RRT-PCR with the recommended method of the European Community Reference Laboratory for Avian Influenza

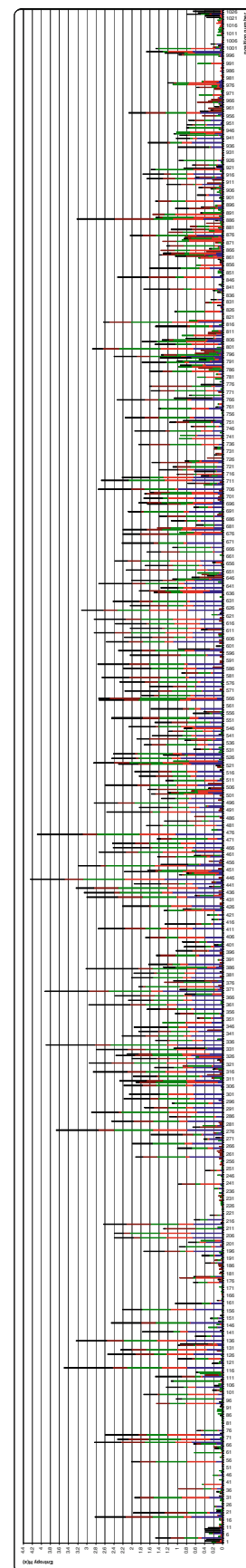
The SVIP-MP assay was compared to the recommended method of the European Community Reference Laboratory (CRL) for Avian Influenza [4, 16]. In the first approach, seven dilution orders of the CZE/H3N8 RNA standard were prepared in three replicates and analysed on the LightCycler 480 instrument. In the second approach, a panel of 62 AI isolates were amplified in two replicates on the Rotorgene 6000 platform, and the mean  $C_t$  values were compared. These assays were performed in two different laboratories.

## Results

### Search for conserved regions in the MP sequence

Integration of the entropy values calculated separately for each MP sequence group into the main entropy plot revealed considerable variation of the IA virus MP segment

**Fig. 2** Main entropy plot of the IA virus MP segment visualised as an assembled column diagram. The entropy values for each position of the MP segment were calculated separately for five sequence groups: avian (blue), human (red), swine (green), equine (brown) and others (black). The partial column height is proportional to the entropy values observed within a given sequence group. The overall variation per position resulted from the assembly of the partial columns



at the nucleotide level (Fig. 2). The main entropy plot further indicated that the positions with elevated nucleotide variation were scattered through the entire segment, with the main stretch of variable sites localised within the central part of the plot, generally delimited by positions 259–819. This variable domain was interrupted by short, up to eight-nucleotide-long conserved sequences. Longer conserved stretches with  $H(x)$  values very close or equal to zero were only found within the termini of the segment enclosed by positions 1–258 and 820–1027. The SVIP-MP assay was designed by fitting the primers and the probe into the strongly conserved positions of the 1–258 region.

#### Evaluation of the primers and probe and estimation of the inclusivity of the assay

The primers and probe selected were evaluated by both bioinformatics analysis and experimental mutations of primer sequences. Bioinformatic analysis of the SVIP-MP primers and probe sequences exhibited full complementarity ranging from 97.07 to 100% of MP strains deposited in the international database (Table 2).

As shown in Table 2, the MP sequences of 0.29 and 0.43% of human strains, 0.18 and 0.17% of pandemic influenza A (H1N1) strains, 0.7 and 1.97% of avian strains, 2.93 and 2.59% of swine strains and 1.93 and 0% of strains of other origin had mutations within the forward and reverse primer binding site, respectively. The ability of the assay to detect these MP sequences was tested with mutated primers mimicking the observed mutations. The SVIP-MP reaction set up with the mutated FX and RX primers was able to detect the CZE/H3N8 RNA standard in all combinations, including primers with mutations localised at the very 3' end.

On the other hand, the octanucleotide SVIP-MP-UPL104 probe was apparently too short to tolerate any mutations within the probe-binding region of the MP segment. However, sequence alignments revealed a low degree of variation in this region as well, i.e. 0.5% in human, 0.16% in pandemic influenza A (H1N1), 0.25% in avian, 1.11% in swine and 0.85% in equine sequence pools, which suggests that these strains may escape detection. To test this possibility, the only IA virus strain in our repository known to hold a single point mutation within the probe-binding region, A/duck/Czechoslovakia/56 (H4N6), was examined. As expected, this mutation was deleterious for probe binding and the A/duck/Czechoslovakia/56 (H4N6) strain escaped detection in the real-time format of the reaction. However, agarose gel electrophoresis revealed a strong band for PCR amplicon, suggesting that the detection of the viral strain was dependent solely on the probe sequence. Overall, we found 21 human, 1 pandemic influenza A (H1N1), 10 avian, 6 swine and 1 equine IA virus strains with mutations in the probe-binding region, which predicts that these strains apparently remain undetectable in this assay (Supplementary Table S3).

In a summary, our results of bioinformatics analysis predicted that the SVIP-MP assay developed in the present study was able to detect 99.5% of human, 99.84% of pandemic influenza A (H1N1), 99.75% of avian, 98.89% of swine, 99.15% of equine and 100% of IA viruses of other origin. The list of IA virus strains with mutations within the regions of interest (primers and probe-binding regions) encompassed a diverse set of IA viruses, which did not show any significant connections or relationships in terms of IA virus subtype, year of isolation, host species or sampling location.

**Table 2** Determination of the initial detection range of the SVIP-MP RRT-PCR assay

	Avian			Human			Pandemic H1N1			Swine			Equine			Others		
	<i>F</i>	<i>R</i>	<i>P</i>	<i>F</i>	<i>R</i>	<i>P</i>	<i>F</i>	<i>R</i>	<i>P</i>	<i>F</i>	<i>R</i>	<i>P</i>	<i>F</i>	<i>R</i>	<i>P</i>	<i>F</i>	<i>R</i>	<i>P</i>
No. of informative sequences per region of interest <sup>#</sup>	4,050	4,125	4,122	4206	4,240	4,245	561	621	625	513	542	542	116	118	118	156	166	166
No. of strains with one and more mutations per region of interest <sup>‡</sup>	28	81	10	12	18	21	1	1	1	15	14	6	0	0	1	3	0	0
Initial inclusivity (%)	<b>99.30</b>	<b>98.03</b>	<b>99.75</b>	<b>99.71</b>	<b>99.57</b>	<b>99.50</b>	<b>99.82</b>	<b>99.83</b>	<b>99.84</b>	<b>97.07</b>	<b>97.41</b>	<b>98.89</b>	<b>100</b>	<b>100</b>	<b>99.15</b>	<b>98.07</b>	<b>100</b>	<b>100</b>

Reference date for sequence download: August 20, 2009

*F* forward primer, *R* reverse primer, *P* probe

<sup>#</sup> Due to the large variation in MP sequence length, the inclusivity was calculated by using the number of MP sequences that fully cover the primer- and probe-binding regions

<sup>‡</sup> The influenza A virus strains with mutations within the probe-binding region of the MP segment are listed in Table S3 (electronic supplementary material)

Evaluation of the SVIP-MP assay

The analytical sensitivity of the assay revealed consistent detection of approximately five RNA copies/reaction. The standard curve indicated a linear correlation ( $R^2 > 0.99$ ) with efficiency values of at least 1.93 (96.5%, slope  $-3.51$ ), obtained in two independent runs (Supplementary Figure F1).

The specificity and cross-reactivity of the SVIP-MP RRT-PCR assay were tested using 24 avian and mammal viruses and 27 pathogenic or ubiquitous bacteria (Supplementary Table S2), and these samples all yielded negative results. Only IA virus isolates were detected; no other viruses or bacteria yielded a specific detection signal. In addition, no detection signal was observed with 42 IA-virus-negative specimens of avian and mammal origin.

The assay exhibited satisfactory intra- and inter-assay reproducibility, as reflected in the low SD and RSD % values (Table 3).

Comparison of the SVIP-MP RRT-PCR with the recommended method of the European Community Reference Laboratory for Avian Influenza

Comparison of the SVIP-MP RRT-PCR with the recommended method of the CRL for Avian Influenza using an in vitro-transcribed RNA standard (diluted from  $10^7$  to  $10^1$  copies/ $\mu$ l) revealed relatively similar sensitivity, with deviations in the SVIP-MP  $C_t$  values ranging from  $-0.8$  to  $-1.5$ .

In contrast, detailed comparison performed on a panel of 62 IA strains revealed strain-, real-time-platform-, and laboratory-dependent variations (data not shown). In this case, however, the SVIP-MP RRT-PCR showed higher sensitivity for the majority of AIV strains.

Evaluation of the SVIP-MP assay using laboratory strains and field samples

The diagnostic quality of the SVIP-MP assay was tested on a panel of 320 IA virus strains of different origin (Supplementary Table S1). All of the IA virus strains tested were successfully detected, indicating that the assay provided 100% efficiency using the panel of analysed viruses. Moreover, analysis of the same IA virus strains in

different laboratories showed consistent results without discrepancies.

To demonstrate the real ability of the SVIP-MP assay to detect IA viruses in field specimens, a collection of 3,423 clinical samples was tested. The  $C_t$  cutoff was adjusted to 36. The surveillance resulted in the detection of various IA virus subtype combinations: H1N1, H2N2, H3N8, H4N6, H5N1, H5N2, H5N3, H6N5, H7N9, H9N2 and H11N9, and two equine H3N8.

Sporadically, non-sigmoidal curve generation appearing at the end of the amplification ( $C_t \geq 38$ ) was observed, especially in surveillance studies. However, the anomalous curve shape and weak signal intensity made them clearly distinguishable from the true positive signal. Nevertheless, these artificial curves were irreproducible, and reanalysis, either by the SVIP-MP or the CRL-recommended method, gave negative results.

Discussion

The ongoing evolution of the IA virus, which generates mutations in the viral genome, requires continuous improvements in molecular detection techniques employing RT or RRT-PCR with primers and probes selected from evolutionarily highly conserved regions. There are several assays based on in vitro amplification of the MP fragment of the IA virus genome [3–5, 9, 11, 16, 18, 21]. Most of them were developed after careful analysis, but some are not completely universal for the detection of all IA virus strains. We focused on the development and evaluation of an RRT-PCR assay for universal detection of IA virus that is suitable for detection the virus in various avian, human and other mammal species and attempted to prove the assay’s universality, both in a bioinformatic and experimental manner.

The primers and the probe were selected on the basis of extensive analysis of variation in the MP segment of the IA virus at the nucleotide level by aligning all of the MP sequences of the ISD database, comprising more than 10,000 sequences.

The results revealed considerable genetic variation along the entire MP segment, which supports previous work [8, 20], but our analysis was based on much broader sequence data. Although the primers and the probe were

**Table 3** Intra- and inter-assay variation of the SVIP-MP assay

Standard concentration (RNA copies/reaction)	Intra-assay variation		Inter-assay variation	
	Mean $C_t \pm$ SD	%RSD	Mean $C_t \pm$ SD	%RSD
$10^2$	29.77 $\pm$ 0.17	0.568	29.49 $\pm$ 0.27	0.919
$10^4$	21.59 $\pm$ 0.07	0.321	21.04 $\pm$ 0.12	0.591
$10^6$	14.16 $\pm$ 0.03	0.196	14.38 $\pm$ 0.14	0.978

selected independently of the previously published primer and probe sequences, when compared with the published oligonucleotides [3–5, 9, 11, 16, 18, 21], the SVIP-MP forward and reverse primer motifs were found within the probe sequence used by Spackman and colleagues [16], and the reverse primer overlapped with the reverse primer sequence described by Ward and colleagues [18]. The probe sequence used in our study was not recognised previously. The combination of sequences for both primers and the hybridization probe used in our study was unique.

To detect as high a proportion of known MP sequences as possible, the lengths of the oligonucleotides in the present study were noticeably below the primer and probe lengths generally applied in PCR techniques [13]. However, this approach ensured over 97% complementarity of MP sequences for the SVIP-MP-F and SVIP-MP-R primers and more than 98.9% for the SVIP-MP-UPL104 probe. In addition, short oligonucleotides decrease the probability that mutations will appear with their binding sites during the ongoing evolution of the MP segment.

On the other hand, the short primers and probe may significantly increase the possibility of anomalous priming, i.e. cross-reactivity, which can result in false positive results. To minimize these undesirable effects, the annealing/hydrolysis step of the assay was optimised to 60°C, since the calculated  $T_m$  values of the SVIP-MP-F and SVIP-MP-R primers were still high, reaching 69.2 and 67.6°C. The short LNA-modified octanucleotide probe was originally designed by the manufacturer to anneal at 60°C.

The quality examination of the primers and probe as well as optimization of the SVIP-MP assay confirmed the high sensitivity, specificity, reproducibility and universality of the present diagnostic method. The sensitivity of our assay, estimated using an *in vitro*-transcribed RNA standard, was comparable with that of RRT-PCR assays developed in other laboratories [4, 16]; however, virus-strain-, real-time-platform- and laboratory- dependent variations were observed. Consistently, the best results were obtained on the Rotorgene 6000 instrument, where, in our hands, the SVIP-MP RRT-PCR showed higher sensitivity for the majority of AIV strains in comparison with the recommended method of the European Community Reference Laboratory for Avian Influenza.

When the specificity of the assay was tested using viral and bacterial samples and negative clinical material, the assay detected only IA viruses of different origin with a very low background signal in negative samples.

To further evaluate the SVIP-MP RRT-PCR, the inclusivity of the assay was assessed as a measure of universality. We defined the inclusivity as a quantitative expression of the portion of MP segments within the nucleotide sequence collection known to date that can be theoretically detected by the assay. The bioinformatic

analysis predicted that sequences for the forward and reverse primers match over 97% of the over 10,000 IA virus sequences tested. Thus, the sequence complementarity was not absolute, even at the strongly conserved positions. Such IA virus strains were highly suspected of being escape mutants in the SVIP-MP assay. To test our assay for universality at the experimental level we used an original approach. Due to experimental limitations, mutated primers mimicking the original mutations were synthesized rather than preparing mutated MP sequences. In spite this experimental shift, we expect that this mechanism may act vice versa. The experiments confirmed that all mutated primers amplified the RNA standard successfully, but deviations in  $C_t$  were observed. These deviations ranged from 3.1, 4.2 and 3.6  $C_t$  values for mutated forward and 3, 2.9 and 2.8 for mutated reverse primers, respectively, observed at three dilutions ( $10^2$ ,  $10^4$  and  $10^6$  copies/reaction). Taken together, we conclude that the primers developed in our study had 100% inclusivity on the basis of retrospective sequence data.

Detailed analysis of IA virus strains with mutations within the regions of interest indicated sporadic variation without any correlation to the year of isolation, subtype, host species or sampling location. Nevertheless, the strains carrying mutations within the probe-binding region apparently escaped detection. Therefore, the inclusivity of the SVIP-MP assay is determined solely via probe complementarity.

The universality of the primers and the specificity of the probe were confirmed by experiments on a broad collection of 320 IA virus strains of various reservoirs, geographic origin and collection date. Since all of the strains were detected successfully, the predicted inclusivity of the SVIP-MP assay was confirmed. The newly developed assay was successfully integrated into the IA virus surveillance system in the Czech Republic during 2007–2009 and in the investigation of three avian H5N1 outbreak, one avian H7N9 outbreak, and one equine H3N8 outbreak. Finally, the assay was used for the detection of 30 cases of pandemic influenza A (H1N1) in the Czech Republic.

In summary, our study indicates that the SVIP-MP assay based on TaqMan real-time RT-PCR is characterized by excellent parameters and provides a reliable, universal and unambiguous tool for the detection of IA viruses from avian, human and other mammal species. Since the assay was carefully evaluated and is used in surveillance analysis, we believe that it can become a method of choice for surveillance or confirmation purposes in other influenza virus diagnostic laboratories.

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