CoVetLab Project Final Report 2014-2015

Project Number: CF0010

Project Title: Development and validation of molecular tools for sub-typing swine influenza viruses

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Progress Against Project Milestones

Milestone from Project Proposal	Achieved Y/N	Summary of Progress
1. Initial evaluation of existing molecular sub-typing protocols – March 2014.	Y	Achieved - An inventory of existing molecular protocols across all five partners (and two associate partners) was compiled and a panel of swine influenza A viruses (swIAVs) and RNA from the ESNIP3 bank held at APHA was sent out to all participating laboratories for provide data for the initial evaluation of the sub-typing protocols. Agreements to send out the viruses to the partners were granted and MTAs prepared.
2. Parallel comparison of molecular and conventional sub-typing protocols – April 2014.	Y	Achieved – a panel of virus isolates and corresponding RNA samples was assembled by APHA and dispatched to five partner laboratories and the two associate partner laboratories. The panel was tested by the protocols employed by all seven partners and the results sent back to APHA for analysis.
3. Design of alternative/ additional primers/probe sets in RRT-PCR – January 2015.	Y	Parallel comparison of molecular and conventional sub-typing protocols from the previous milestone (2.) demonstrated that alternative/ additional primers/probe

		sets did not need to be designed for RRT-PCR.
4. Evaluation of alternative/additional primers/probe sets in RRT-PCR – February 2015.	Y	The most appropriate existing primers/probe sets were selected for further validation work at the Project Meeting held at DTU on 4 February 2015.
5. Initial evaluation of Fluidigm Dynamic Array – February 2015.	Y	Assays were unified and tested under equal conditions at DTU. Pilot tests on the Fluidigm platform were completed.
6. Formal and final ring trial of new and existing protocols among partners – March 2015.	Y (partial)	A protocol for a final ring trial, using sets of the most appropriate primer/probes was discussed during the Project Meeting held at DTU on 4 February 2015, and by e-mail afterwards. A suitable protocol for comparison with the potential for publication in a scientific journal was agreed on and work started by the individual partners on the panel from 2014. Not all results were available at the time of the Final Report but the work will continue. Results were submitted from Anses, SVA, DTU and APHA.

Executive Summary

Swine influenza (SI) is a major economically important disease in all of the major swine-producing countries of Europe and four major sub-types (avH1N1, H1N1pdm09, H1N2 and H3N2) of swine influenza A viruses (swIAVs) plus various reassortant combinations have been isolated in pigs. Diagnostic tests and advanced technologies capable of rapidly identifying the sub-types of the circulating strains rapidly within the pig population are therefore critically important. Traditional serological methods for HA and NA sub-typing are laborious, costly, time-consuming and interpretation of results suffers from non-specific cross-reactions of test antigens and lack of any information about the genetic constellation of genes of the viruses. Furthermore, conventional assays were not able to detect novel viruses or distinguish the emergence of new reassortants. Harmonization of diagnostic tools for detection of swIAV was initiated through the concerted action: “European surveillance network for influenza in pigs (ESNIP) 3”, running from 2010 until 2013 to fill gaps in surveillance for influenza viruses in pigs. A deliverable of the ESNIP 3 concerted action was the organization of an inter-laboratory comparison test to evaluate the ability of partners to detect swIAV genome by reverse transcription polymerase chain reaction (RT-

PCR) assays. Most of the participating partners were able to detect IAV genome, especially using RT-PCR to target the matrix (M)-gene. Real-time RT-PCR (RRT-PCR) formats could potentially improve the sensitivity and speed of RT-PCR methodology for swIAV sub-typing and overcome shortcomings with conventional RT-PCR formats. This led to the selection of a panel of representative swIAV isolates from the ESNIP 3 virus bank for a parallel comparison of the molecular and conventional sub-typing protocols employed across the partners in an initial ring trial. The best-performing primers/probe sets and amplification protocols could be used in RRT-PCR assays specific for detection of H1 of the different genetic lineages (H1av, H1hu, H1pdm), for H3, for N1 (N1av and N1pdm) and for N2. Parallel comparison of the sub-typing protocols from the initial ring trial confirmed that alternative/additional primers/probe sets did not need to be designed for RRT-PCR as new variants, for example of N1, were emerging locally. The selected primer/probe sets and amplification protocols were used by the partners in a formal and final ring trial on the same panel of isolates from the ESNIP 3 bank. While further optimization will probably be necessary in each laboratory depending on materials and equipment, these harmonized protocols enabled partners to achieve successful, sensitive and specific identification of HA and NA genes of swIAVs from enzootic European lineages. The assays provided fast results and enabled a semi-quantitative assessment to be made of virus loads. Furthermore, in a separate work package, the different sub-typing assays were modified to run on the integrated fluidic circuits (IFCs) technology (Fluidigm) platform at DTU which has the advantage of being cheaper and much faster. Initial tests with the platform revealed that the assays can be run under similar conditions with comparable sensitivity but more validation is needed before reliable results can be obtained. In conclusion, primer/probe sets have been evaluated for improved detection and differentiation of the European swIAV sub-types in simplex or duplex RRT-PCR assays with differentiation between the pandemic and avian-like H1N1 virus strains as well as identification of reassortant viruses. These RRT-PCR assays are a valuable resource for swIAV sub-typing. The harmonized protocols will enable partners to sub-type previously uncharacterized swIAV strains without having to depend on virus isolation and sequencing. Importantly, however, the protocols will also facilitate sub-typing of swIAV RT-PCR-positive samples from clinical material which were negative by virus isolation.

Methods and Results

Initial evaluation of existing molecular sub-typing protocols:

At the start of the project, molecular sub-typing tools for swIAVs already existed as conventional multiplex RT-PCR assays for differentiating European swIAV sub-types H1N1, H1N2 and H3N2 at IZSLER (Chiapponi et al., 2012) and H1N1pdm09 (Slomka et al., 2010; Pol et al., 2011) were being used by partners in the ESNIP 3 consortium (Simon et al., 2014). Real-time tetraplex and duplex assays for differentiation of HA and NA sub-types respectively, had also been disseminated from FLI to five ESNIP 3 partners for evaluation in a multi-centre study during the lifetime of the ESNIP 3 concerted action. Building on this, an updated evaluation of existing molecular sub-typing protocols from all five partners and both associate partners was carried out. Each institute was asked to provide an inventory of the real-time and conventional RT-PCR protocols, including both multiplex and simplex formats, used in their laboratory. The full inventory of the protocols was then compiled and disseminated to partners (**Appendix 1**).

The inventory revealed a wide variety of primers/probe sets and protocols, including both multiplex and simplex formats, already being used by the partners (**Appendix 1**). Two of the partners; namely, SVA and the APHA routinely ran only the 'perfect match' and H1-118 assays (for generic detection of swIAV and H1N1pdm09, respectively).

Parallel comparison of molecular and conventional sub-typing protocols already in use at the start of the project:

A panel of seven representative swIAV isolates (**Table 1**) was chosen from the ESNIP 3 virus bank held at the APHA to compare the molecular sub-typing protocols already being employed across the partners. RNA was extracted from the egg-amplified virus using the QIAmp viral RNA BioRobot kit customized for the APHA in conjunction with a Universal BioRobot (QIAGEN, Manchester, United Kingdom). For each isolate, both the extracted nucleic acid and the corresponding egg-amplified isolate were sent to all partners for the initial evaluation of both the locally-employed RNA extraction and RT-PCR amplification protocols.

Table 1: Composition of swIAV panel for comparison of molecular sub-typing protocols in the initial ring trial

Isolate No.	Isolate name	Isolate sub-type
1	A/swine/England/34627/12	H1N2
2	Negative	N/A
3	A/swine/France/Ille et Vilaine/0187/11	H1N1
4	A/swine/France/Morbihan/0213/11	rH1N2
5	A/swine/Italy/302749-2/2010	H3N2
6	A/swine/Italy/122573/2012	H1N2
7	A/swine/England/502321/1994	H3N2
8	A/swine/Germany/Ebbinghof-12991-1/2011	H1N1pdm

A Project Meeting was held at DTU on 4th February 2015 for presentation and discussion of the initial ring trial results. The results from each partner are summarised in **Table 2** and presented in detail in **Appendix 2**.

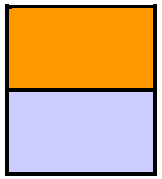
In summary, all partners correctly determined the swIAV sub-type/lineage of each isolate from the mini-panel although in some cases, nucleotide sequencing was used to complement the RT-PCR protocols in order to conclude the sub-type of the isolates. The correct determination of the swIAV sub-type/lineages was achieved by IZSLER using their conventional RT-PCR protocol. Notably, Anses reported successful assay performance with the FLI primers for H1av, H1hu, H3, N1 and N2, slightly modified and adapted to French strains (**Appendix 2**).

Design of alternative/additional primers/probe sets:

The comparison of the molecular sub-typing protocols confirmed that alternative/additional primers/probe sets did not need to be designed for RRT-PCR. The best-performing existing primers/probe sets were selected among those initially designed and provided by FLI and the accompanying protocols were selected for further validation work.

Table 2: Summary of the results from the parallel comparison of molecular sub-typing protocols already in use at the start of the project in the initial ring trial

		H1N2	Negative	H1N1	rH1N2	H3N2	H1N2	H3N2	pH1N1
1	**CVI**	SIV pos and H1N2	SIV neg	SIV pos and H1N1	SIV pos and rH1N2	SIV pos and H3N2	SIV pos and H1N2	SIV pos and H3N2	SIV pos and pH1N1
2	**FLI**	SIV pos and H1N2	SIV neg	SIV pos and H1N1	SIV pos and rH1N2	SIV pos and H3N2	SIV pos and H1N2	SIV pos and H3N2	SIV pos and pH1N1
3	**IZSLER**	SIV pos and H1N2	SIV neg	SIV pos and H1N1	SIV pos and rH1N2	SIV pos and H3N2	SIV pos and H1N2	SIV pos and H3N2	SIV pos and pH1N1
4	**SVA**	SIV pos and H1N2	SIV neg	SIV pos and H1N1	SIV pos and rH1N2	SIV pos	SIV pos and H1N2	SIV pos and H3N2	SIV pos and pH1N1
5	**DTU**	SIV pos and H1N2	SIV neg	SIV pos and H1N1	SIV pos and rH1N2	SIV pos and H3N2	SIV pos and H1N2	SIV pos and H3N2	SIV pos and pH1N1
6	**ANSES**	SIV pos and H1N2	SIV neg	SIV pos and H1N1	SIV pos and rH1N2	SIV pos and H3N2	SIV pos and H1N2	SIV pos and H3N2	SIV pos and pH1N1
7	**APHA**	SIV pos	SIV neg	SIV pos	SIV pos	SIV pos	SIV pos	SIV pos	SIV pos and pH1N1



- perfect result

Evaluation of alternative/additional primers/probe sets in a formal and final ring trial of new/modified protocols among partners:

The selected protocols for further evaluation comprised three simplex RRT-PCRs for H1av, H1hu and H3 amplification and one duplex RRT-PCR for N1/N2 amplification (**Table 3**):

- Simplex RRT-PCR 1: Amplification of H1 gene from avian-like swine H1N1 viruses > H1 of avian origin (H1av).

- Simplex RRT-PCR 2: Amplification of H1 gene from human-like reassortant swine H1N2 viruses > H1 of human origin (H1hu).

- Simplex RRT-PCR 3: Amplification of H3 gene from human-like reassortant swine H3N2 viruses > H3 of human origin (H3).

- RRT-PCR 4 (duplex): Amplification of N1 and/or N2 genes from avian-like swine H1N1, human-like reassortant swine H1N2 and human-like reassortant swine H3N2 viruses.

The sequences (5' – 3') and orientation of the primers/probe sets are shown in **Table 3** and **Appendix 3.** These RRT-PCRs were suitable for the specific identification of HA and NA genes of swIAVs from enzootic European lineages, except those from the pandemic-like lineage (H1pdm, N1pdm). H1pdm and N1pdm specific assays were run on M-gene positive samples prior to the RT-PCRs described below. It was agreed that the H1pdm- and N1pdm-specific assays used by the different partners were equivalent and did not need any modification or improvement.

Standard Operating Procedures were provided by Anses to the other partners (**Appendix 3**).

Table 3: Swine influenza virus sub-type specificity and sequence of the primers/probe sets selected for the final ring trial of new/modified protocols among partners

Primer/probes target and name	Orientation	Sequence (5' - 3')
Simplex real-time RT-PCR 1: H1 of avian origin (H1av)		
H1_Swine_avi_Fw	Forward	gaaggrggatggacaggaatga
H1_Swine_avi_Rv-2	Reverse	caattahtgarttcactttgttgc
H1_Swine_avi_HEX		HEX-tctggttacgcagcwgatcagaaaa-BHQ1
Simplex real-time RT-PCR 2: H1 of human origin (H1hu)		
H1[N2]_Swine_Fw	Forward	gaggggrtggaccggaatgatagatgga[i]5tggttatcatca
H1[N2]_Swine_Rv	Reverse	acctacagctgtgaattgagtgttcatyttntcg[i]5agagttcacct
H1[N2]_Swine_Fw-2	Forward	ggatggtacggttatcatca
H1[N2]_Swine_Rv-2	Reverse	tttcgatcacagaattcacct
H1[N2]_Swine_FAM		FAM-cagggatctggctatgctgcagayc-BHQ2
Simplex real-time RT-PCR 3: H3 of human origin		
H3_Swine_Fw	Forward	cttgatggrgmaaaytgcaca
H3_Swine_Rv	Reverse	ggcacatcatawgggtaaca
H3_Swine_CY5		CY5-ctctattgggrgaccctcaytgtga-BHQ2
Duplex real-time RT-PCR 4: N1/N2		
N1_pan_Fw	Forward	agrccttgyttctgggttga
N1_pan_Rv	Reverse	accgtctggccaagacca
N1_pan_FAM		FAM-atytggacyagtgggagcagcat-BHQ1
N2_pan_Fw	Forward	agtctggtggacytcaaayag
N2_pan_Rv	Reverse	ttgcgaaagcttatatagvcatga
N2_pan_HEX		HEX-ccatcaggccatgagcctgwwccata-BHQ1

The assay protocols were adapted by Anses from the initial protocol suggested by FLI: some fluorescent dyes were modified; some primers and probes initially provided were not selected and incorporated. Running conditions were modified in order to carry out the four RRT-PCR assays at the same time with the same chemistry (**Table 4**).

Table 4: Thermal amplification profile

Temperature	Time	Step
45°C	15 min	Reverse transcription
95°C	2 min	*Taq* polymerase activation
95°C	15 sec	42 cycles
56°C	1 min	

Beyond the initial objectives of this project, Anses also initiated an adaptation of these RT-PCR assays to high throughput analyses using LightCycler®1536 technology (data not shown). The first results were satisfactory and were presented

to CoVetLab partners at the project meeting held at DTU. Further development is in progress.

It was not desirable to completely standardize the parameters, notably RNA extraction protocol and PCR kits, across all partners. Therefore, it was proposed that each partner implement the selected protocols and subsequently test eight decimal dilutions (10-fold) of the strains supplied from the ESNIP 3 bank (**Table 1**). Partners therefore made dilutions of the seven strains provided by the APHA in the ring trial (the negative sample number 2 was not included).

Partners initially extracted RNA from the 200 µl of virus stock provided by the APHA and then made the serial dilutions. As different extraction protocols were used across the partners, the main objective was to evaluate the "extraction + PCR" efficiency at the laboratory level.

Because of standardisation of PCR-protocols by each individual partner, it was not desirable to use different PCR kits from what was already in use, so each used their own PCR kit. However, this allowed a comparison of the protocols used in the respective institutes to be made on a standardised set of samples. "Extraction + PCR" efficiency could therefore be studied in the respective laboratory, starting with the same set of samples and the final results compared across all laboratories.

The results submitted from Anses, SVA, DTU and APHA with a brief summary of the assay methodology used are shown in the separate tables in **Appendix 4.** Although optimization of the test protocols and conditions will probably be necessary in the respective laboratories, each partner successfully used the selected primers/probe sets and adapted the Anses protocol to detect and sub-type the seven swIAV samples in the panel, demonstrating similar limits of detection with the decimal dilutions of each isolate. The "extraction + PCR" efficiency was effective in each laboratory.

Initial evaluation of Fluidigm Dynamic Array

At DTU, all existing RRT-PCR assays were adjusted for sub-typing of swIAVs by PCR in nanolitre volumes using the integrated fluidic circuits (IFCs) technology

(Fluidigm). This system uses less sample and reagent volumes compared to standard PCR platforms and a single microfluidic device to achieve high-quality consistent results. Using the Biomark HD system, up to 96 different samples can be tested in 96 different assays providing 9.216 reactions in less than 3 hours. Once fully validated, this system can replace all existing assays and will save time, resource and manpower at a competitive price.

The work plan was divided into three separate parts:

1. Primer and probe sequences from existing RRT-PCR assays for all European sub-types of swIAV provided by all partners including several in-house assays were compared, adjusted and purchased.
2. Validation of the different assays were tested by RRT-PCR on appropriate, known swIAV isolates adjusted to the profile selected for the Fluidigm assay (2 or 3 step thermos cycling, melting temperature and elongation temperature and time for each step).
3. Preliminary tests of the adjusted assays were run on the Fludigm platform.

All existing assays were adjusted to run using similar conditions (same extraction kit, same cDNA preparation, similar annealing, extension temperature and same number of themocycles). Optimisation was carried out by testing the assays on the Rotogene platform and the best-performing assays under these conditions were selected for the Fludigm chip. These included several assays for detection of all European HA and NA subtypes and as well as several pan-influenza A assays targeting the M and NP proteins.

The system was tested in one run on the Fludigm platform using the established assays and using a one-step (cDNA and PCR) protocol without pre-amplification. Results were disappointing in that none of the assays gave the expected outcome on known positive samples. The set up will be changed from a one-step to a two-step protocol. Tests of several different protocols for cDNA preparation combined with or without pre-amplification are ongoing. Further optimisation will be performed depending on the results of these tests.

Discussion

At the outset, sub-typing RT-PCR protocols for swIAVs were needed by the partners to complement their virus isolation and nucleotide sequencing activities. Sub-typing of swIAV RT-PCR-positive samples which were negative by virus isolation was also required. This project addressed this requirement through the provision and harmonization of molecular diagnostic tools for detection and sub-typing of swIAV initiated through the ESNIP 3 concerted action. IZSLER and Anses had successfully been using a conventional multiplex assay but only with samples yielding Ct values < 30 by the swIAV generic M-gene RRT-PCR.

Following the parallel comparison of existing protocols used by partners in the initial ring trial, Anses reported successful assay performance with the FLI primers for H1av, H1hu, H3, N1 and N2, slightly modified and adapted to French strains (unpublished data). Thus for the formal and final ring trial of new/modified protocols made on a standardised set of samples from the ESNIP 3 virus bank, H1av, H1hu and H3 primer sets were used in parallel as simplex protocols, and N1 and N2 primer sets used in a duplex assay (Anses having previously used validated commercial kits for H1pdm and N1pdm detection). Even though further optimization and validation will probably be necessary in each laboratory depending on materials and equipment, these harmonized protocols enabled partners to achieve successful, sensitive and specific identification of HA and NA genes of swIAVs from enzootic European lineages. The H1pdm- and N1pdm-specific assays used by all partners were equivalent and did not need any modification or improvement. Success was also achieved by Anses with a prototype high throughput protocol involving the LightCycler®1536.

For each partner, the value of cooperation in this project was visible on several levels. Firstly, the exchange of protocols allowed partners to critically review their own protocols for swIAV detection and sub-typing. Secondly, partners were able to test more different methods and viruses than on an individual laboratory level. Following the completion of ESNIP 3 in October 2013, the project has enabled

consortium partners to retain connectivity on both a personal and laboratory level. Exchange of the project results between partners allowed further evaluation of the reliability of the tests in the broadest sense, with the potential for each partner to upgrade existing molecular sub-typing tools to enable fast and reliable differentiation between the most important HA and NA lineages of influenza in European swine.

The harmonized protocols will enable partners to sub-type previously uncharacterized swIAV strains without having to depend on virus isolation and sequencing. More than this, Anses have sub-typed swIAVs from clinical material that tested positive by M-gene RRT-PCR which was negative by virus isolation. Not all viruses in the original clinical material were sub-typed; this depended on the viral genome load in the sample so sub-typing of some M-gene PCR-positive samples may be unsuccessful (totally or partially). However, the availability of the harmonized protocols means that a higher proportion of swIAVs can be sub-typed than in the past. In turn, this might reduce the need for serological reagents requiring *in vivo* production. It is envisaged that the sub-typing protocols will pave the way for the validation of Next-Generation Sequencing protocols for full characterization of swIAVs in Europe, including the internal gene segments.

Recommendations

1. The FLI H1av, H1hu and H3 primer sets to be used in parallel as simplex protocols, and N1 and N2 primer sets used in a duplex assay for sub-typing of European swIAVs.

2. The H1pdm- and N1pdm-specific assays used by the different partners are equivalent and do not need any modification or improvement.

3. Different PCR kits rather than common kits can be used as, with the selected primers/probe sets in the final ring trial, “Extraction + PCR” efficiency was comparable between laboratories.

It is anticipated that at least one peer-review publication will be submitted from this project. Additional outputs from oral and/or poster presentations at the Influenza Conference 2015 (St. Hilda's College, Oxford, 8-10 September, 2015) and at the 24th International Pig Veterinary Society Congress 2016 (Dublin, 7-10 June, 2016 in partnership with the 8th European Symposium of Porcine Health Management) are also expected.

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Final report agreed by Head of Seedcorn

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Copy returned to project manager: Yes/No
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Appendix 1: Detailed inventory of swIAV RT-PCR protocols used by CoVetLab partners at the start of the project

Anses	Methods:			Forward primer sequence 5´-3´	Reverse primer sequence 5´-3´	Probe sequence 5´-3´	Published reference(s)	Validated in-house (Yes or No)	Target Gene	Amplicon size	Modifications to primers and/or probe (eg. LNA, MGB)	Reporter dye and quencher
	1	**Amplification of H1 gene from avian-like swine H1N1 viruses: H1 of avian origin (H1av)**	**H1_Swine_avi (forward from set 1 and reverse from set 2)**	gaaggrggatggacaggaatga	caattahtgarttcactttgttgc	HEX-tctggttacgcagcwgatcagaaaa-BHQ1	Harder et al., personal communication	**Yes**	**H1av**	**138**	We do not use the two sets of primers initially proposed by Harder et al. (only the forward primer from set 1 and the reverse primer from set 2).	**Reporter= HEX, Quencher=BHQ1**
		RT-PCR Chemistry (kit/enzyme):		**Go Taq Probe 1-Step RT-qPCR System (#A6120)-PROMEGA**								
		Thermo profile:		**Step 1 : 45°C 15min/Step2: 95°C, 2 min/Step 3: 95°C,15 seconds, 56°C 1 min (42 repeats). Read fluorescence with HEX channel at each repeat.**								
			Extraction methodology:	**Rneasy Mini kit (Qiagen), Nucleospin RNA Kit (Macherey Nagel), Nucleospin Virus Kit (Macherey Nagel), Nucleospin 8RNA Kit (Macherey Nagel) can be used for RNA extraction**								
				Extraction is performed on 200 µl of virus strain supernatants, nasal swab supernatants, BALF or allantoïc fluids. RNA extraction from tissues (lung) is performed on 20-30mg. Details are given in the RNA extraction kit's notice.								

	Methods:			Forward primer sequence 5´-3´	Reverse primer sequence 5´-3´	Probe sequence 5´-3´	Published reference(s)	Validated in-house (Yes or No)	Target Gene	Amplicon size	Modifications to primers and/or probe (eg. LNA, MGB)	Reporter dye and quencher
	2	**Amplification of H1 gene from human-like reassortant swine H1N2 viruses : H1 of human origin (H1hu)**	**H1[N2]_Swine (set 1)**	gaggggggrtggaccggaatgatagatgga[i]$_5$tggttatcatca	acctacagctgtgaattgagtgttcatyttntcg[i]$_5$agagttcacct	ROX-cagggatctggctatgctgcagayc-BHQ2	Harder et al., personal communication	**Yes**	**H1hu**	**169**	No	**Reporter= ROX, Quencher=BHQ2**
			H1[N2]_Swine (set 2)	tttcgatcacagaattcacct	tttcgatcacagaattcacct							
		RT-PCR Chemistry (kit/enzyme):		**Go Taq Probe 1-Step RT-qPCR System (#A6120)-PROMEGA**								
		Thermo profile:		**Step 1 : 45°C 15min/Step2: 95°C, 2 min/Step 3: 95°C,15 seconds, 56°C 1 min (42 repeats). Read fluorescence with ROX channel at each repeat.**								
			Extraction methodology :	**Rneasy Mini kit (Qiagen), Nucleospin RNA Kit (Macherey Nagel), Nucleospin Virus Kit (Macherey Nagel), Nucleospin 8RNA Kit (Macherey Nagel) can be used for RNA extraction**								
				Extraction is performed on 200 µl of virus strain supernatants, nasal swab supernatants, BALF or allantoïc fluids. RNA extraction from tissues (lung) is performed on 20-30mg. Details are given in the RNA extraction kit's notice.								

	Methods:			Forward primer sequence 5´-3´	Reverse primer sequence 5´-3´	Probe sequence 5´-3´	Published reference(s)	Validated in-house (Yes or No)	Target Gene	Amplicon size	Modifications to primers and/or probe (eg. LNA, MGB)	Reporter dye and quencher
	3	**Amplification of H3 gene from human-like reassortant swine H3N2 viruses : H3 of human origin (H3)**	**H3_Swine**	cttgatggrgmaaaytgcaca	ggcacatcatawgggtaaca	CY5-ctctattgggrgaccctcaytgtga-BHQ2	Harder et al., personal communication	**Yes**	**H3**	**119**	No	**Reporter= CY5, Quencher= BHQ2**
		RT-PCR Chemistry (kit/enzyme):		**Go Taq Probe 1-Step RT-qPCR System (#A6120)-PROMEGA**								
		Thermo profile:		**Step 1 : 45°C 15min/Step2: 95°C, 2 min/Step 3: 95°C,15 seconds, 56°C 1 min (42 repeats). Read fluorescence with CY5 channel at each repeat.**								
			Extraction methodology :	**Rneasy Mini kit (Qiagen), Nucleospin RNA Kit (Macherey Nagel), Nucleospin Virus Kit (Macherey Nagel), Nucleospin 8RNA Kit (Macherey Nagel) can be used for RNA extraction**								
				Extraction is performed on 200 µl of virus strain supernatants, nasal swab supernatants, BALF or allantoïc fluids. RNA extraction from tissues (lung) is performed on 20-30mg. Details are given in the RNA extraction kit's notice.								
	Additional assay information:			**These three sets of primers are used separatly in monoplex assays. Multiplex assays have been attempted but did not give satisfactory results.**								

DTU	Methods:			Forward primer sequence 5´-3´	Reverse primer sequence 5´-3´	Probe sequence 5´-3´	Published reference(s)	Validated in-house (Yes or No)	Target Gene	Amplicon size	Modifications to primers and/or probe (eg. LNA, MGB)	Reporter dye and quencher
	1			AGT TCA AGC CGG AAA TAG CA	CCC GGC TCT ACT AGT GTC CA	CCC AAA GTG AGG GAT CAA GAA GGG A	unpublished (confidential use)	Yes	HA		none	FAM-BHQ1
		RT-PCR Chemistry (kit/enzyme):		**1: QIAGEN OneStep RT-PCR Kit**								
		Thermo profile:		**1: : 50C, 20 min - 95C, 15 min - 45X(95C, 15 sec; 55ºC, 60 sec, aquire to FAM/Green) on RotorgeneQ**								
			Extraction methodology :	**1: QIAGEN RNeasy Mini Kit (lung tissue, masal swabs), QIAampViral RNA Mini Kit (oral fluid), QIAsymphony RNA Kit (nasal swabs).**								
	Additional assay information:			**1: Specific for H1N1pdm09 HA gene.**								

SVA	Methods:			Forward primer sequence 5´-3´	Reverse primer sequence 5´-3´	Probe sequence 5´-3´	Published reference(s)	Validated in-house (Yes or No)	Target Gene	Amplicon size	Modifications to primers and/or probe (eg. LNA, MGB)	Reporter dye and quencher
	1			AGA TGA GTC TTC TAA CCG AGG TCG	TGC AAA GAC ACT TTC CAG TCT CTG	FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA	Slomka, M.J. et al. (2010) Influenza and Other Respiratory Viruses, 4: 277-293.	Yes	Matrix		none	FAM-TAMRA
		RT-PCR Chemistry (kit/enzyme):										
		Thermo profile:										
			Extraction methodology:									
	Additional assay information:			1. Perfect match assay								
	Methods:			Forward primer sequence 5´-3´	Reverse primer sequence 5´-3´	Probe sequence 5´-3´	Published reference(s)	Validated in-house (Yes or No)	Target Gene	Amplicon size	Modifications to primers and/or probe (eg. LNA, MGB)	Reporter dye and quencher
	2							Yes				
		RT-PCR Chemistry (kit/enzyme):										
		Thermo profile:										
			Extraction methodology:									
	Additional assay information:			2: WHO recommended pH1N1 assay								

CVL	Methods:			Forward primer sequence 5´-3´	Reverse primer sequence 5´-3´	Probe sequence 5´-3´	Published reference(s)	Validated in-house (Yes or No)	Target Gene	Amplicon size	Modifications to primers and/or probe (eg. LNA, MGB)	Reporter dye and quencher
	1			CTTCTAACCGAGGTCGAAACGTA	CACTGGGCACGGTGAGC	TCAGGCCCCCTCAAAGCCGA	Loeffen WL, de Vries RP, Stockhofe N, van Zoelen-Bos D, Maas R, Koch G, Moormann RJ, Rottier PJ, de Haan CA. Vaccination with a soluble recombinant hemagglutinin trimer protects pigs against a challenge with pandemic (H1N1) 2009 influenza virus. Vaccine. 2011 Feb 11;29(8):1545-50.	Yes (not for pigs)	M	204		FAM / BHQ1
		RT-PCR Chemistry (kit/enzyme):		QuantiFast Probe RT-PCR Kit								
		Thermo profile:		15 min 50°C, 5 min 95°C, 5 sec 95°C , 15 sec 58°C , 20 sec 72°C (45x cycles)								
			Extraction methodology:	MagNa Pure 96 / MagNA Pure LC total Nucleic Acid Isolation Kit								
	Additional assay information:											

APHA	Methods:			Forward primer sequence 5´-3´	Reverse primer sequence 5´-3´	Probe sequence 5´-3´	Published reference(s)	Validated in-house (Yes or No)	Target Gene	Amplicon size	Modifications to primers and/or probe (eg. LNA, MGB)	Reporter dye and quencher
	1			AGA TGA GTC TTC TAA CCG AGG TCG	TGC AAA GAC ACT TTC CAG TCT CTG	FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA	Slomka, M.J. et al. (2010) Influenza and Other Respiratory Viruses, 4: 277-293.	Yes	Matrix		none	FAM-TAMRA
		RT-PCR Chemistry (kit/enzyme):		Qiagen Onestep RT-PCR kit								
		Thermo profile:		30 min 50°C, 15 min 95°C, 40 cycles of 10 secs 95°C followed by 20 secs 60°C								
			Extraction methodology:	QIAamp viral RNA BioRobot kit customised for APHA								
				Universal BioRobot (Qiagen)								
	Additional assay information:			1. Perfect match assay								
	Methods:			Forward primer sequence 5´-3´	Reverse primer sequence 5´-3´	Probe sequence 5´-3´	Published reference(s)	Validated in-house (Yes or No)	Target Gene	Amplicon size	Modifications to primers and/or probe (eg. LNA, MGB)	Reporter dye and quencher
	2			AAT GCC GAA CTG TTG GTT CT	CAA TTT CCT TGG CAT TGT TTT	FAM- CTG GCT TCT TAC CTT TTC ATA TAA GTT CTT C	Slomka, M.J. et al. (2010) Influenza and Other Respiratory Viruses, 4: 277-293.	Yes	H1		Antisense probe with an internal T nucleotide that links through a sidechain to BHQ1. Additionally, the 3' terminal C nucleotide is modified with a dideoxy sugar as ddC to prevent extension of	FAM-BHQ1

											the probe.	
		RT-PCR Chemistry (kit/enzyme):		Qiagen Onestep RT-PCR kit								
		Thermo profile:		30 min 50°C, 15 min 95°C, 40 cycles of 10 secs 95°C, 30 secs at 54°C and 10 secs 72°C								
			Extraction methodology:	QIAamp viral RNA BioRobot kit customised for APHA								
				Universal BioRobot (Qiagen)								
	Additional assay information:			**H1-118 assay for detection of pandemic (H1N1)pdm09**								

Appendix 2: Initial ring trial results for parallel comparison of molecular and conventional sub-typing protocols already in use by CoVetLab partners

Partner	Sample type submitted by APHA	Sample number and identification		Swine influenza virus gene amplified by RT-PCR								Conclusion (swIAV subtype/ lineage)
				M	H1pdm	N1pdm	H1av	H1hu	H3	N2	N1	
Anses (France)	Virus strains produced by APHA and extracted at Anses	1.	H1N2	13.25	>45	>45	>42	15.78	>42	14.48	>42	**$H1_{hu}N2$**
		2.	Negative	**34.73/36.94/ >45**	>45	>45	>42	>42	>42	>42	>42	**Negative**
		3.	H1N1	13.25	>45	>45	16.49	>42	>42	>42	14.05	**$H1_{av}N1$**
		4.	rH1N2	13.04	>45	>45	14.32	>42	>42	14.44	>42	**reassortant $H1_{av}N2$**
		5.	H3N2	15.49	>45	>45	>42	>42	15.5	14.41	>42	**H3N2**
		6.	H1N2	15.6	>45	>45	>42	19.9	>42	17.66	>42	**$H1_{hu}N2$**
		7.	H3N2	14.15	>45	>45	>42	>42	27.26	15.88	>42	**H3N2**
		8.	H1N1pdm	12.64	14.4	13.87	>42	>42	>42	>42	13.26	**H1N1pdm09**
	RNA extracts provided by APHA	1.	H1N2	13.27	>45	>45	>42	19.47	>42	18.32	>42	**$H1_{hu}N2$**
		2.	Negative	**39.16/>45**	>45	>45	>42	>42	>42	>42	>42	**Negative**
		3.	H1N1	13.31	>45	>45	21.01	>42	>42	>42	17.82	**$H1_{av}N1$**
		4.	rH1N2	13.48	>45	>45	20.01	>42	>42	18.76	>42	**reassortant $H1_{av}N2$**
		5.	H3N2	16.13	**>45/38.74**	**36.59**	>42	>42	18.53	17.9	>42	**H3N2** (+ H1N1pdm)
		6.	H1N2	14.26	>45	>45	>42	21.74	>42	19.39	>42	**$H1_{hu}N2$**
		7.	H3N2	13.8	**37.19/34.95**	**36.66**	>42	>42	29.02	18.92	>42	**H3N2** (+ H1N1pdm)
		8.	H1N1pdm	13.25	16.19	15.41	>42	>42	**36.92/ 35.87**	>42	18.4	**H1N1pdm09** (+H3)
Anses methodology – brief summary				Commercial kit	Commercial kit		In-house	In-house	In-house	In-house	In-house	
DTU (Denmark)	Virus strains produced by APHA and extracted at DTU	1.	H1N2	14.37	Negative			Detected		N2hu		**$H1_{hu}N2$**
		2.	Negative	Negative	Negative							**Negative**
		3.	H1N1	14.57	Negative		Detected				N1av	**$H1_{av}N1$**
		4.	rH1N2	15.13	Negative		Commercial kit			N2hu		**reassortant $H1_{av}N2$**
		5.	H3N2	12.77	Negative				Detected	N2sw		**H3N2**
		6.	H1N2	16.36	Negative			Detected		N2hu		**$H1_{hu}N2$**
		7.	H3N2	13.58	Negative				Detected	N2hu		**H3N2**
		8.	H1N1pdm	12.81	13.04					N1pdm09		**H1N1pdm09**
	RNA extracts provided by APHA	1.	H1N2	14.66	Negative			Detected		N2hu		**$H1_{hu}N2$**
		2.	Negative	Negative	Negative							**Negative**
		3.	H1N1	13.8	Negative						N1av	**$H1_{av}N1$**

		4.	rH1N2	14.8	Negative			N2hu			**reassortant $H1_{av}N2$**
		5.	H3N2	13.72	**38.11 (?)**		Detected	N2sw			**H3N2**
		6.	H1N2	15.54	Negative	Detected		N2hu			**$H1_{hu}N2$**
		7.	H3N2	15.59	**35.01 (?)**		Detected	N2hu			**H3N2**
		8.	H1N1pdm	13.71	13.04			N1pdm09			**H1N1pdm09**
SVA (Sweden)	Virus strains produced by APHA and extracted at SVA	1.	H1N2	15.8	No Ct						**H1N2**
		2.	Negative	No Ct	No Ct						**Negative**
		3.	H1N1	17	No Ct						**H1N1**
		4.	rH1N2	16.6	No Ct						**H1N2**
		5.	H3N2	23.4	No Ct						**Influenza A (mix?)**
		6.	H1N2	15.9	No Ct						**H1N2**
		7.	H3N2	18.1	No Ct						**H3 (N2)**
		8.	H1N1pdm	15.4	15.3						**pH1Na**
	RNA extracts provided by APHA	1.	H1N2	14.6	No Ct						**H1N2**
		2.	Negative	No Ct	No Ct						**Negative**
		3.	H1N1	15.4	No Ct						**H1N1**
		4.	rH1N2	15.5	No Ct						**H1N2**
		5.	H3N2	21.2	**35.9 (?)**						**Influenza A (mix?)**
		6.	H1N2	16.6	No Ct						**H1N2**
		7.	H3N2	16.1	No Ct						**H3 (N2)**
		8.	H1N1pdm	14.8	14.8						**pH1Na**
CVL (The Netherlands)	Virus strains produced by APHA and extracted at CVL	1.	H1N2								
		2.	Negative								
		3.	H1N1								
		4.	rH1N2								
		5.	H3N2								
		6.	H1N2								
		7.	H3N2								
		8.	H1N1pdm								
	RNA extracts provided by APHA	1.	H1N2	13.91	No Ct	39.56	No Ct	+	No Ct		**"Common" European H1N2**
		2.	Negative	No Ct	No Ct	No Ct	No Ct	-	No Ct		**Negative**
		3.	H1N1	15.24	No Ct	38.5	No Ct	-	28.5		**$H1_{av}N1$**
		4.	rH1N2	15.26	No Ct	31.98	No Ct	+	No Ct		**reassortant $H1_{av}N2$**
		5.	H3N2	14.67	No Ct	No Ct	38.93	+	No Ct		**European H3N2**
		6.	H1N2	16.37	No Ct	42.71	No Ct	+	No Ct		**rH1N2**
		7.	H3N2	15.2	No Ct	No Ct	No Ct	+	No Ct		**European H3N2**
		8.	H1N1pdm	13.46	27.21	No Ct	No Ct	-	No Ct		**H1N1pdm09**
CVL methodology						CVL H1 PCR	CVL H3 PCR	N2 PCR (gel-based)	N1 PCR		

FLI (Germany)	Virus strains produced by APHA and extracted at FLI	1.	H1N2	15.76	Negative		Negative	18.22	Negative	16.97	Negative	**SIV $H1_{hu}N2$**
		2.	Negative	Negative	Negative		Negative	Negative	Negative	Negative	Negative	**Negative**
		3.	H1N1	17.03	Negative		15.58	Negative	Negative	Negative	15.02	**SIV $H1_{av}N1$**
		4.	rH1N2	15.29	Negative		14.5	Negative	Negative	16.39	Negative	**SIV $H1_{av}N2$**
		5.	H3N2	15.95	Negative		Negative	Negative	14.3	16.99	Negative	**SIV H3N2**
		6.	H1N2	17.31	Negative		Negative	Negative	Negative	17.88	Negative	**SIV H1N2**
		7.	H3N2	16.48	Negative		Negative	Negative	Negative	18.91	Negative	**IAV H3N2**
		8.	H1N1pdm	15.09	14.27		Negative	Negative	Negative	Negative	14.58	**IAV H1N1pdm**
	RNA extracts provided by APHA	1.	H1N2									
		2.	Negative									
		3.	H1N1									
		4.	rH1N2									
		5.	H3N2									
		6.	H1N2									
		7.	H3N2									
		8.	H1N1pdm									
FLI qRT-PCR methodologies run >				NP generic	H1pdm		H1av	H1hu	H3	N2	N1	
IZSLER (Italy)	Virus strains produced by APHA and extracted at IZSLER	1.	H1N2	14.4	Negative		Negative	**Positive**	Negative	**Positive**	Negative	**$H1_{hu}N2$**
		2.	Negative	Negative	Negative		Negative	Negative	Negative	Negative	Negative	**Negative**
		3.	H1N1	15.7	Negative		**Positive**	Negative	Negative	Negative	**Positive**	**$H1_{av}N1$**
		4.	rH1N2	15.1	Negative		**Positive**	Negative	Negative	**Positive**	Negative	**$H1_{av}N2$**
		5.	H3N2	16.5	Negative		Negative	Negative	Negative	**Positive**	Negative	**H3N2**
		6.	H1N2	19.4	Negative		Negative	**Positive**	Negative	**Positive**	Negative	**H1huN2**
		7.	H3N2	15.9	Negative		Negative	Negative	**Positive**	**Positive**	Negative	**H3N2**
		8.	H1N1pdm	15.1	16		Negative	Negative	Negative	Negative	**Positive**	**H1N1pdm**
	RNA extracts provided by APHA	1.	H1N2	15.5	Negative		Negative	**Positive**	Negative	**Positive**	Negative	**$H1_{hu}N2$**
		2.	Negative	Negative	Negative		Negative	Negative	Negative	Negative	Negative	**Negative**
		3.	H1N1	16.1	Negative		**Positive**	Negative	Negative	Negative	**Positive**	**$H1_{av}N1$**
		4.	rH1N2	16.3	Negative		**Positive**	Negative	Negative	**Positive**	Negative	**$H1_{av}N2$**
		5.	H3N2	17.7	Negative		Negative	Negative	Negative	**Positive**	Negative	**H3N2**
		6.	H1N2	17.4	Negative		Negative	**Positive**	Negative	**Positive**	Negative	**$H1_{hu}N2$**
		7.	H3N2	16.5	Negative		Negative	Negative	**Positive**	**Positive**	Negative	**H3N2**
		8.	H1N1pdm	15.2	15.7		Negative	Negative	Negative	Negative	**Positive**	**H1N1pdm**
IZSLER methodology >				Matrix	H1pdm		H1av	H1hu	H3	N2	N1	Multiplex
APHA (UK)	RNA extracts provided by APHA	1.	H1N2	17.15								
		2.	Negative	No Ct								
		3.	H1N1	19.8								
		4.	rH1N2	19.53								
		5.	H3N2	23.43								
		6.	H1N2	20.74								
		7.	H3N2	19.24								
		8.	H1N1pdm	16.2								
APHA methodology >				Matrix								

Appendix 3: Standard Operating Procedures provided by Anses for evaluation of alternative/additional primers/probe sets in a formal and final ring trial of new/modified protocols among partners

Ploufragan-Plouzané Laboratory
Swine Virology Immunology Unit
National Reference Laboratory for Swine Influenza

Standard Operating Procedure

(MO.GRIP.17)

Molecular sub-typing of European Swine Influenza Viruses:

- ✓ **3 simplex real-time RT-PCRs for $H1_{av}$, $H1_{hu}$, H3 amplification**
- ✓ **1 duplex real-time RT-PCR for N1/N2 amplification**

Purpose

These RT-PCRs are used for the specific identification of HA and NA genes of Swine Influenza Viruses from enzootic European lineages, except those from the pandemic-like lineage (H1pdm, N1pdm). H1pdm and N1pdm specific assays (commercial kits validated by the French NRL) are run on M-gene positive samples prior to the RT-PCRs described below.

***RT-PCR N°1*: Amplification of H1 gene from avian-like swine H1N1 viruses > H1 of avian origin ($H1_{av}$)**

***RT-PCR N°2*: Amplification of H1 gene from human-like reassortant swine H1N2 viruses > H1 of human origin ($H1_{hu}$)**

***RT-PCR N°3*: Amplification of H3 gene from human-like reassortant swine H3N2 viruses > H3 of human origin (H3)**

***RT-PCR N°4 (duplex)*: Amplification of N1 and/or N2 genes from avian-like swine H1N1, human-like reassortant swine H1N2 and human-like reassortant swine H3N2 viruses**

These assays are run on RNA extracts that are obtained from biological samples (nasal swab supernatants, lung tissue…) or from virus isolates (viruses propagated on MDCK cells or embryonated chicken eggs).

***Warning**: to avoid any loss of specificity, these assays have to be preferentially run on RNA extracts that were detected positive in M-gene RT-PCR in obtaining Ct value of 20-30. In case M-gene RT-PCR Ct value is below 20, a dilution has to be performed before running molecular subtyping.*

References

Primers and probes have been designed and provided by FLI, Germany (Harder et al., personal communication).
The methods have been adapted from the initial protocol suggested by FLI: some fluorescent dyes have been modified; some primers and probes initially provided have not been selected and incorporated. Running conditions have been modified in order to carry out the 4 RT-PCRs at the same time, with the same chemistry.

Primers and probes

Primers and probes	**5' > 3'**
RT-PCR N°1_ H1_Swine-avi	
H1_Swine_avi_Fw	gaaggrggatggacaggaatga
H1_Swine_avi_Rv-2	caattahtgarttcactttgttgc
H1_Swine_avi_HEX	HEX-tctggttacgcagcwgatcagaaaa-BHQ1
RT-PCR N°2_ H1[N2]_Swine	
H1[N2]_Swine_Fw	gaggggrtggaccggaatgatagatgga[i]$_5$tggttatcatca
H1[N2]_Swine_Rv	acctacagctgtgaattgagtgttcatyttntcg[i]$_5$agagttcacct
H1[N2]_Swine_Fw-2	ggatggtacggttatcatca
H1[N2]_Swine_Rv-2	tttcgatcacagaattcacct
H1[N2]_Swine_FAM	FAM-cagggatctggctatgctgcagayc-BHQ2
RT-PCR N°3 H3_Swine	
H3_Swine_Fw	cttgatggrgmaaaytgcaca
H3_Swine_Rv	ggcacatcatawgggtaaca
H3_Swine_CY5	CY5-ctctattgggrgaccctcaytgtga-BHQ2
RT-PCR N°4 (duplex) N1/N2	
N1_pan_Fw	agrccttgyttctgggttga
N1_pan_Rv	accgtctggccaagacca
N1_pan_FAM	FAM-atytggacyagtgggagcagcat-BHQ1
N2_pan_Fw	agtctggtggacytcaaayag
N2_pan_Rv	ttgcgaaagcttatatagvcatga
N2_pan_HEX	HEX-ccatcaggccatgagcctgwwccata-BHQ1

Primer/probe mix preparation

- For 200 reactions each

RT-PCR N°1_ H1_Swine-avi		
Oligo name	Stock Solution (µM)	Volume (µL)
H1_Swine_avi_Fw	200	20
H1_Swine_avi_Rv-2	200	20
H1_Swine_avi_HEX	100	5
Nuclease free water		355
RT-PCR N°2_ H1[N2]_Swine		
Oligo name	Stock Solution (µM)	Volume (µL)
H1[N2]_Swine_Fw	200	20
H1[N2]_Swine_Rv	200	20
H1[N2]_Swine_Fw-2	200	20
H1[N2]_Swine_Rv-2	200	20
H1[N2]_Swine_FAM	100	7
Nuclease free water		313
RT-PCR N°3 H3_Swine		
Oligo name	Stock Solution (µM)	Volume (µL)
H3_Swine_Fw	100	40
H3_Swine_Rv	100	40
H3_Swine_CY5	100	5
Nuclease free water		315
RT-PCR N°4 (duplex) N1/N2		
Oligo Name	Stock solution (µM)	Volume (µL)
N1_pan_Fw	100	40
N1_pan_Rv	100	40
N1_pan_FAM	100	5
N2_pan_Fw	100	40
N2_pan_Rv	100	40
N2_pan_HEX	100	5
Nuclease free water		630

Chemistry
Kit = GoTaq® Probe 1-Step RT-qPCR System, A6120 (Promega)

This reagent contains a passive reference dye (CXR dye) in a separate tube: this passive reference is not used in the mastermix.
The reverse transcriptase (Go Script RT mix for One Step RT-qPCR) is packed in a separate tube.

The Taq Polymerase is already mixed into the *Go Taq Probe 2X qPCR Master mix.*

Thermocycler

These assays have been validated on BioRad Chromo4 and Stratagene/Agilent MX3005P.

Mastermix setting up for RT-PCR N°1, N°2 or N°3

Pipetting order	**Components**	**1 x**	**N x**
1.	Go Taq Probe 2 X qPCR Master mix	12,5 µl	
2.	Go Script RT mix for 1 Step RT-qPCR	1 µl	
3.	Primer/probe mix (*H1_Swine-avi* or H1[N2]_Swine or *H3_Swine*)	2 µl	
4.	Nuclease free water	4.5 µl	
	Total volume	**20 µl**	
Addition of template RNA			
5.	RNA sample or Positive Control (PC) or NTC	5 µl	
	Total volume reaction mix	**25 µl**	

Mastermix setting up for RT-PCR N°4 (duplex) N1/N2

Pipetting order	**Components**	**1 x**	**N x**
1.	Go Taq Probe 2 X qPCR Master mix	12,5 µl	
2.	Go Script RT mix for 1 Step RT-qPCR	1 µl	
3.	Primer/probe mix *duplex N1/N2*	4 µl	
4.	Nuclease freee Water	2.5 µl	
	Total volume	**20 µl**	
Addition of template RNA			
5.	RNA sample or Positive Control (PC) or NTC	5 µl	
	Total volume reaction mix	**25 µl**	

Temperature profile

Temperature	Time	Step
45°c	15 min	Reverse transcription
95°c	2 min	Taq polymerase activation
95°c	15 sec	42 cycles
56°c	1 min	

Amplicon detection

Reads of three fluorescence channels are required.

Necessity to take care of plate setup, as two probes are read in the FAM channel and two others are read in the HEX channel!

FAM for H1[N2]_Swine_FAM and N1_pan_FAM detection
HEX for H1_Swine_avi_HEX and N2_pan_HEX detection
CY5 for H3_Swine_CY5 detection

Appendix 4: Results from evaluation of alternative/additional primers/probe sets in a formal and final ring trial of new/modified protocols among partners

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National Reference Laboratory for Swine Influenza
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CoVetLab 2014 – swIAV Molecular sub-typing APHA ring trial - Part 2 (sensitivity evaluation)

Panel of 16 samples, received on 12 June 2014

Virus number	SIV N°3		SIV N°4		SIV N°1		SIV N°6		SIV N°5		SIV N°7		SIV N°8		
RT-PCR assay	H1av	N1	H1av	N2	H1 hu	N2	H1 hu	N2	H3	N2	H3	N2	H1 pdm	N1 pdm	N1
Ct	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dRn)	Ct (dRn)	Ct (dR)
Previous result with undiluted RNA	16,49	14,05	14,32	14,44	15,78	14,48	19,9	17,66	15,5	14,41	27,26	15,88	14,4	13,87	13,26
Dilution 10^{-1}	19.52	17.90	17.87	17.75	18.48	17.79	21.93	20.41	18.13	18.78	28.18	19.58	19.37	18.30	18.29
Dilution 10^{-2}	22.92	21.57	21.39	20.88	21.96	20.52	25.49	24.06	21.61	22.13	31.69	22.86	23.15	21.56	21.24
Dilution 10^{-3}	25.37	24.95	24.22	24.67	25.59	24.49	28.55	27.93	24.89	25.88	35.93	26.34	26.34	25.06	24.40
Dilution 10^{-4}	28.09	27.04	26.88	28.78	28.79	27.94	31.01	*No Ct*	28.27	*No Ct*	*No Ct*	*No Ct*	29.44	28.49	27.25
Dilution 10^{-5}	30.98	*No Ct*	29.43	*No Ct*	32.00	*No Ct*	*No Ct*	*No Ct*	31.72	*No Ct*	*No Ct*	*No Ct*	32.88	31.90	*No Ct*
Dilution 10^{-6}	34.09	*No Ct*	32.83	*No Ct*	*No Ct*	*No Ct*	*No Ct*	*No Ct*	34.53	*No Ct*	*No Ct*	*No Ct*	35.63	34.78	*No Ct*
Dilution 10^{-7}	40.86	*No Ct*	38.59	*No Ct*	*No Ct*	*No Ct*	*No Ct*	*No Ct*	37.01	*No Ct*	*No Ct*	*No Ct*	*No Ct*	38.42	*No Ct*
Dilution 10^{-8}	*No Ct*	*No Ct*	*No Ct*	*No Ct*	*No Ct*	*No Ct*	*No Ct*	*No Ct*	*No Ct*	*No Ct*	*No Ct*	*No Ct*	*No Ct*	*No Ct*	*No Ct*

H1av, H1hu, H3, N1, N2: see Anses SOP (MO.GRIP.17) (**Appendix 3**).
H1pdm, N1pdm: see Pol et al., J Virol. Methods 171: 241-247.

Virus number	SIV#3		SIV#4		SIV#1		SIV#6		SIV#5		SIV#7		SIV#8	
RT-PCR assay	H1av	N1	H1av	N2	H1hu	N2	H1hu	N2	H3	N2	H3	N2	H1pdm*	N1
Ct	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)
Undiluted	16.66	13.90	15.46	13.97	15.28	13.04	16.55	14.63	15.45	12.79	24.47	13.05	14.08	13.85
diluted 10^{-1}	23.56	17.36	19.28	16.78	19.28	17.64	20.34	19.63	18.41	16.38	27.64	17.57	17.54	17.05
diluted 10^{-2}	25.47	21.05	25.47	19.97	24.82	22.87	24.94	22.98	22.7	20.14	31.93	21.66	21.31	21.54
diluted 10^{-3}	27.98	25.12	28.83	23.47	28.66	27.24	29.03	25.82	26.3	24.73	36.2	26.07	24.63	24.99
diluted 10^{-4}	31.03	28.34	32.53	27.78	33.31	31.14	32.54	29.40	31.04	28.09	No CT	32.44	29.14	28.36
diluted 10^{-5}	34.46	No CT	36.24	No CT	39.18	No CT	No CT	No CT	35.66	No CT	No CT	No CT	32.05	No CT
diluted 10^{-6}	39.86	No CT	No CT	No CT	No CT	No CT	No CT	No CT	39.16	No CT	No CT	No CT	35.99	No CT
diluted 10^{-7}	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT	39.18	No CT
diluted 10^{-8}	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT

All assays were performed as described in the ANSES SOP (MO.GRIP.17 – **Appendix 3**), for sample 8 WHO recommended H1pdm assay* was used for H1 detection.

DTU - swIAV sub-typing ANSES protocol

Virus number	SIV # 3		SIV # 4		SIV # 1		SIV # 6		SIV # 5		SIV # 7		SIV # 8	
RT-PCR assay	H1av	N1	H1av	N2	H1 hu	N2	H1 hu	N2	H3	N2	H3	N2	H1 pdm	N1
Ct	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)
Undiluted	16.59	13.89	15.88	13.12	12.93	12.28	15.78	13.8	?	11.93	26.85	11.7	12.84	12.6
diluted 10^{-1}	?	18.29	19.02	16.97	16.76	15.81	19.55	17.47	?	16.16	28.56	15.12	15.64	16.6
diluted 10^{-2}	24.59	21.68	23.54	20.45	20.51	19.98	23.16	21.24	20.97	20.08	34.03	18.91	19.09	20.52
diluted 10^{-3}	28.32	24.65	26.89	23.57	23.91	22.84	26.28	24.53	25.17	24.6	35.99	22.79	22.61	23.49
diluted 10^{-4}	32.25	27.87	30.22	27.51	27.47	26.96	29.27	28.5	33.2	28.63	No Ct	26.09	26.31	27.04
diluted 10^{-5}	34.52	No Ct	33.44	No Ct	No Ct	31.12	No Ct	No Ct	32.61	No Ct	No Ct	31	30.15	No Ct
diluted 10^{-6}	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	36.15	No Ct	No Ct	No Ct	33.71	No Ct
diluted 10^{-7}	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	37.92	No Ct
diluted 10^{-8}	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct

All assays described in the Anses protocol were performed and the results are listed above.

For sample 8, an in house H1pdm assay was used, the N1 results are from the N1pan assay described in the Anses protocol, and NOT N1pdm09-specific.

APHA - CoVetLab sub-typing panel - May 2015

Virus number	SIV#3		SIV#4		SIV#1		SIV#6		SIV#5		SIV#7		SIV#8	
RT-PCR assay	H1av	N1	H1av	N2	H1hu	N2	H1hu	N2	H3	N2	H3	N2	H1pdm	N1
Ct	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)	Ct (dR)
diluted 10^{-1}	22.94	18.52	19.83	18.87	19.44	19.05	17.9	17.14	16.41	19.62	31.8	19.74	21.31	18.5
diluted 10^{-2}	26.16	22.12	22.75	22.13	22.91	21.75	21.88	20.9	19.83	22.13	34.68	22.99	24.66	21.69
diluted 10^{-3}	29.69	25.97	26.32	25.09	26.37	25.99	25.28	24.4	23.48	26.07	38.98	27.87	27.64	25.81
diluted 10^{-4}	32.06	29.05	29.63	29.08	No CT	28.19	30.72	27.2	25.82	2.02	No CT	30.21	30.99	28.34
diluted 10^{-5}	34.99	31.89	32.75	33.3	No CT	31.55	No CT	30.94	29.57	32.36	No CT	34.06	N/T	31.25
diluted 10^{-6}	37.64	No CT	35.54	No CT	No CT	37.8	No CT	35.18	32.69	39.09	No CT	No CT	N/T	34.03
diluted 10^{-7}	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT	35.52	No CT	No CT	No CT	N/T	No CT
diluted 10^{-8}	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT	No CT	N/T	No CT

All tests performed as described in the ANSES SOP (MO.GRIP.17), using Quantifast Probe RT-PCR Kit

H1pdm - tested by H1-118.