DETECTION AND SUBTYPING
OF SWINE INFLUENZA VIRUSES IN CLINICAL SAMPLES
BY THE MEAN OF DEVELOPED MULTIPLEX POLYMERASE
CHAIN REACTION ASSAYS

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Abstract

Multiplex PCR assays that can detect and identify three haemagglutinins and two neuraminidases of three main subtypes: H1N1, H1N2, and H3N2 of swine influenza virus (SIV), circulating in a pig population, were developed. Three oligonucleotide primer sets were evaluated based on the published sequences, with unique sizes characteristic for each subtype. The sequences of each primers were demonstrated to be specific for every subtype of SIV with the cDNA of reference viruses. Furthermore, the assays could detect and subtype up to 10^{-1} dilution of 10^4 $EID_{50}/0.2$ mL of H1N1 and 10^{-1} dilution of 10^2 EID_{50} /0.2 mL of H1N2. For the H3N2 mPCR test, sensitivity was observed in a dilution as low as 10^{-3}, which equals 10 $EID_{50}/0.2$ mL. Conditions for the reactions and reagents concentrations were optimised. The optimal temperature was also ensemble. For all RNA positive samples in the RT-nested-PCR test for influenza A viruses, the mPCR agreed completely. In 19 farms (95% of cases) the H1N1 subtype was determined, and in one farm H3N2 subtype was confirmed. Therefore, these methods could facilitate the rapid and accurate subtyping of influenza A viruses directly from field specimens.

Key words: swine, swine influenza virus, multiplex PCR, subtyping, clinical material.

Swine influenza virus (SIV) is one of the most common factors responsible for pigs respiratory diseases. It is also considered to be an important factor in public health concern. A recent review described about 51 cases of symptomatic human infection with SIV, due to its capability to cross the interspecies barrier. Because the pig is well know as a mixing vessel for avian, human, and swine influenza viruses, and mutations in their genome could occur at a high level, there is a necessity to control infections caused by SIV (12).

Sixteen subtypes of haemagglutinin within the type A influenza virus have been reported, but in swine population HA1 and HA3 have been commonly identified throughout the world (15).

Several diagnostic tests, including virus isolation (VI), immunohistochemistry, antigen-capture ELISA, indirect fluorescent antibody, haemagglutination assay (HA), and haemagglutination inhibition (HI) have been used for the diagnosis of influenza in pigs. Single reverse transcription-polymerase chain reaction (RT-PCR) assay has been reported for the detection of type A influenza viruses in humans (1) and swine (11, 16).

The polymerase chain reaction is an enzyme-driven process through which a specific DNA fragment can be selectively amplified a million-fold *in vitro*. Since its development in the 1980s, the PCR technique has been continually improved and modified to extend its versatility and applicability (4). Despite its powerfulness in a variety of target DNA amplifications, PCR is not yet generally accepted as a definitive method in analytical applications due to the conditions of the amplification process. More sophisticated conceptual variants of the PCR system, like multiplex PCR, containing more than one primer set to amplify more than one fragment of a target DNA, required greater attention and optimisation during its efforts. Those aspects of amplification, by which tiny amount of artefacts or contaminants would simultaneously be amplified, could interfere with the results. The comparison of the PCR tests among laboratories using many different sets of primers should include some critical factors such as the effect of temperature variations, and the importance of a calibration of the thermal cycles on the reproducibility of PCR performances (19).

The purpose of this study was to develop multiplex RT-PCR assays that can identify and subtype H1 and H3, as well as N1 and N2 of SIV, directly in clinical specimens.

Material and Methods

Samples. A total of 297 clinical samples (252 nasal swabs and 45 lung tissue samples), taken from animals demonstrating symptoms of influenza-like respiratory disorders, raised in 30 Polish farms, were used for the isolation of SIV and its genetic material. The samples were first submitted to laboratory examination, including virology (VI in embryonated chicken eggs and HA tests) and genetic study (nested-PCR). Isolates with positive results in the first testing were examined in three multiplex reverse-transcription reaction assays carried out in order to detect HA and NA genes of H1N1, H1N2, and H3N2.

Viruses. Three reference SIV strains of different subtypes: H1N1 (A/Sw/Bel/1/98) with EID_{50} $10^4/0.2$ mL; H1N2 (A/Sw/Eng/96) with EID_{50} $10^2/0.2$ mL and H3N2 (A/Sw/Fl/1/98) with EID_{50} $10^4/0.2$ mL were used for the standardisation of tests as the control material of the study. Ten-fold dilutions of reference strains stock were used for the examination. The dilutions were equivalent to 10^3, 10^2, 10^1, 1, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} EID_{50} of H1N1 and H3N2, whereas for H1N2 titers equal to 10^1, 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} EID_{50} were used. The reaction mixture was incubated at room temperature for 10 min, at 42ºC for 45 min, and at 95 ºC for 5 min and quenched on ice.

Viral RNA and cDNA. RNA was extracted by means of the RNA Total Isolation Kit (A&A Biotechnology, Poland) according to the manufacturer's procedure. Reverse transcription was performed by mixing 5 μl of RNA with 15 μl of RT reaction mixture (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 125 μM each dNTP, 1 unit of RNaze, and 100 units of M-MLV enzyme (Invitrogen). cDNA was synthesised using 50 ng of Random Primers (Invitrogen) in a single reaction.

Primer design. Three oligonucleotide primers sets suitable for multiplex PCR were designed based on the sequences published in NCBI. Selected accessions numbers for the HA and NA genes sequences are described in Table 1. According to the high variability in the virus genome, only the nucleotide information related to the European SIV isolates was taken into consideration. The primers were designed with the aid of the open software package "Primer3" (http://frodo.wi.mit.edu). Conceptually, the primer pairs were designed to amplify the two distinguishable fragments of the gene and to be used long enough for a sequencing in the next step of the virus characterisation.

Next, the stability and ability of the oligomers to form secondary structures (hairloop and primer-dimer structure) was verified using the LaserGene – PrimerSelect package.

PCR condition. The PCR mixes were prepared based on commercial components using thermostabile polymerase Platinium (Invitrogen). To optimise the multiplex PCR, the primer's concentration tested ranged from 5.5 to 50 pmol, and the $MgCl_2$ concentration ranged from 0.5 to 3.5 mM. The annealing temperature was also determined experimentally, with a range from 52.2°C to 62.2°C.

Sensitivity and specificity. The sensitivity was evaluated using serial 10-fold dilutions of the stock of each of the three reference strains. The dilutions were directly used for the RNA extraction. The specificity of the mPCR was assessed by testing the genetic material extracted from the common pathogens responsible for the pulmonary infection in pigs, including porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fiver virus (CSFV), Aujeszky's disease virus (ADV), and bacteria: *Mycoplasma hyopneumoniae (Mhp), Bordetella bronchiseptica (Bp), Pasteurella multocida (Pm),* and *Actinobacillus pleuropneumoniae (App)*. Additionally, two avian influenza virus (AIV) strains: H7N4 A/Turkey/95/95 and H5 A/Robin/Poland/119-128/02 were used.

Results

In the PCR tests, all the primer sets used for all of the selected genes permitted the amplification of the fragments of the expected sizes: HA1 (H1N1) – 780 pb, HA1(H1N2) – 360 pb, H3 – 516 bp, N1 – 514 bp, and N2 – 791 bp. Amplicons with a molecular size different than that expected were not obtained in any of the samples tested. None of the other swine viruses, nor the bacterial pathogens, showed any amplification (data not shown), which indicates that the elaborated tests were specific.

Table 1
The multiplex PCR primers used to subtype the swine influenza viruses

| Genes	Gene Bank sequence's accession number	Primer's name	Oligonucleotyde sequences
HA1 (H1N1)	AJ51781	H1K1F	ACCATGCTAACAATTCCACA
		H1K1R	CATCCGACATCATAATTCCA
HA1 (H1N2)	AF085416	H12KF	CTGTGGCTGACGGAGAAAGA
		H12KR	TGAGACGATGATTCCTGACC
HA3	AJ293930	H3-31F	CATTTTATGTCTGGTTCTTGG
		H3-547R	TATTCCGCATCCCTGTTG
NA1	CY010582	N1F	TGAAATACAATGGCATAATAAC
		N1R	GGATCCCAAATCATCTCAAA
NA2	CY010566	N2F	GGAAAAGCATGGCTGCAT
		N2R	GTGCCACAAAACACAACAAT
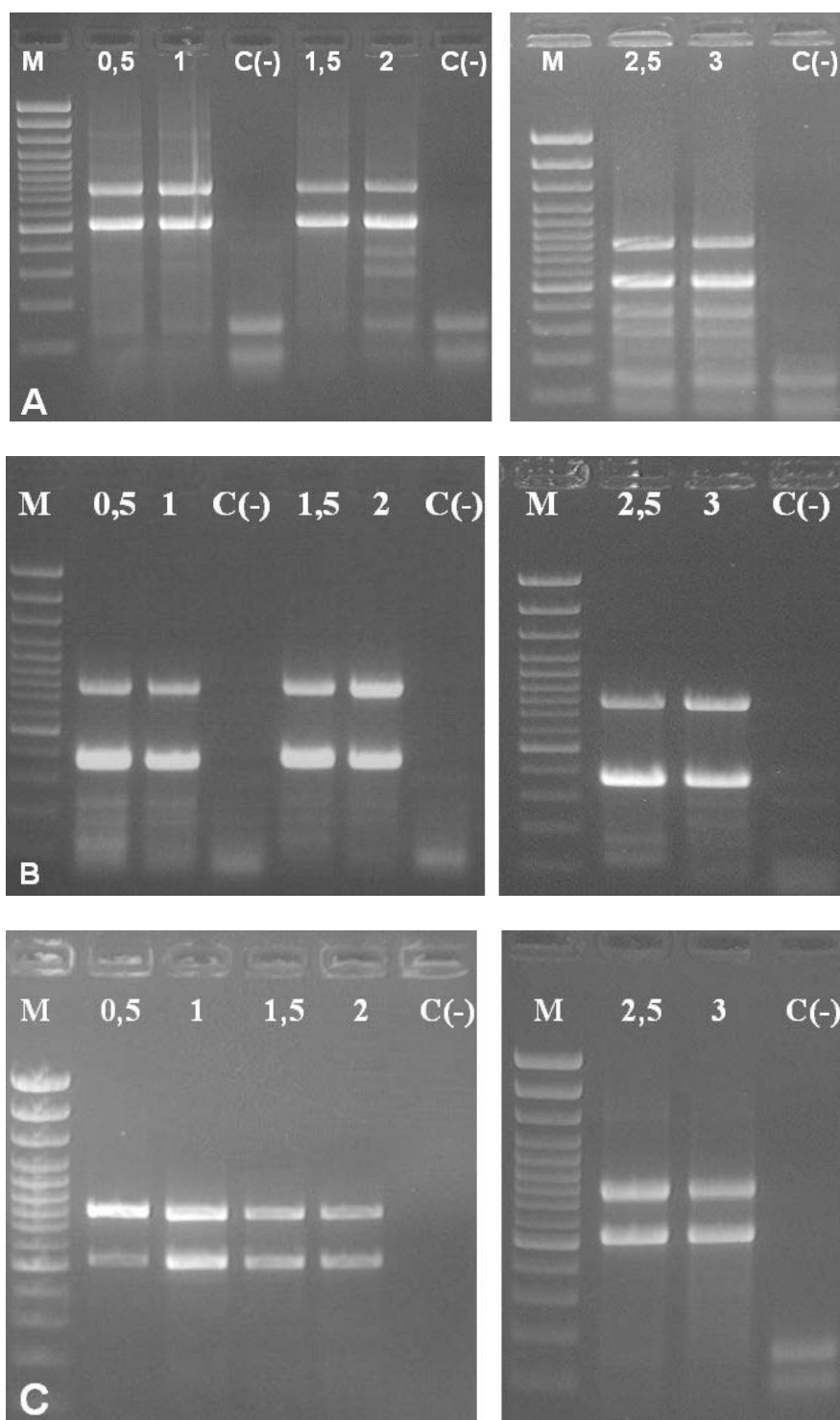
Fig. 1. Impact of the Mg^{2+} concentration in the mPCR for subtyping: H1N1 (A), H1N2 (B), and H3N2 (C).
M – 100 pb DNA ladder (Fermantas), K(-) – negative control; numbers correspond to the concentration of Mg^{2+} in mM
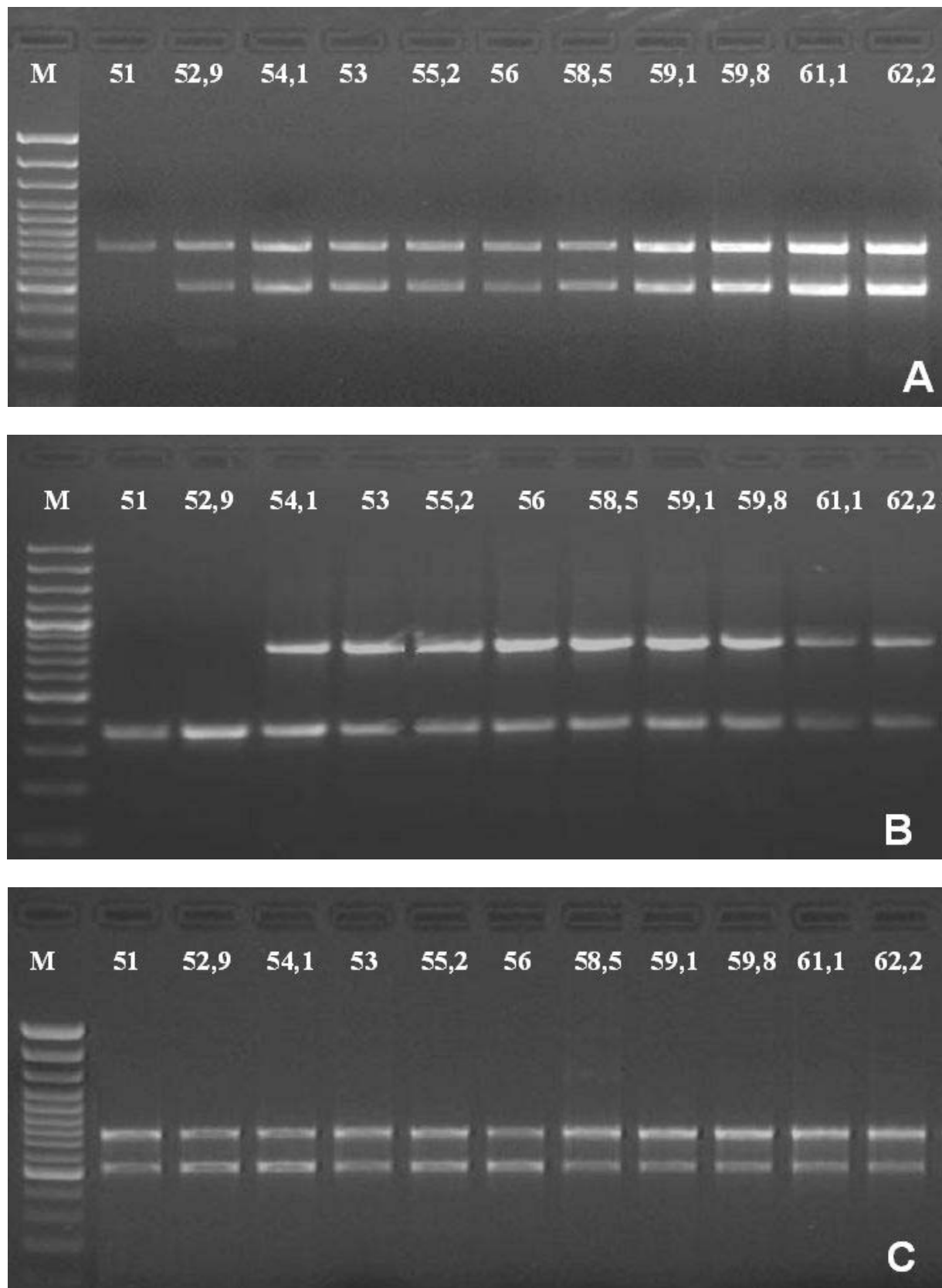
Fig. 2. Examination of the optimal temperature in the H1N1 (A), H1N2 (B), and H3N2 (C) mPCR tests; the numbers correspond to the temperature.
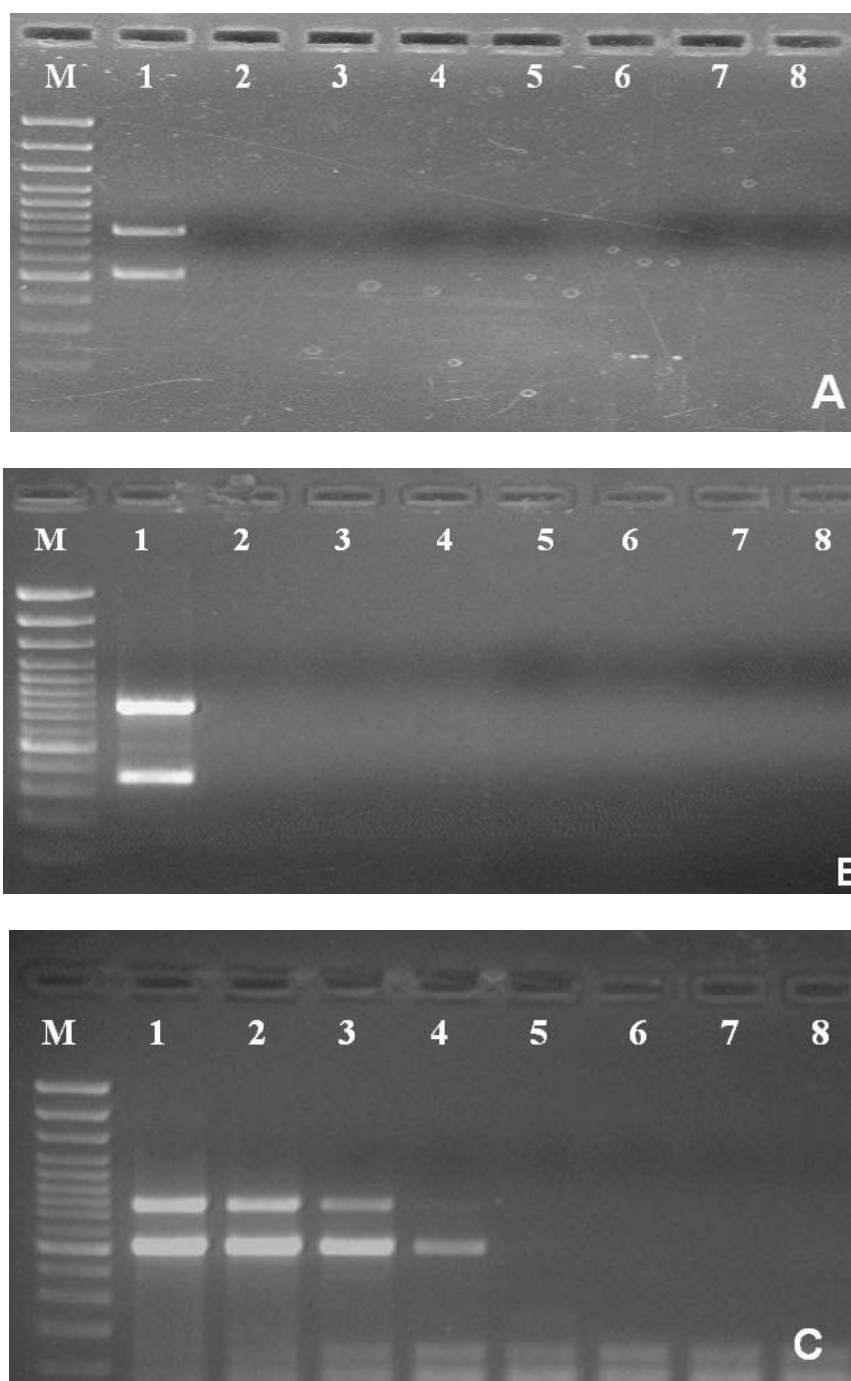
Fig. 3. Electrophoretic profiles of the DNA amplification products in the mPCR sensitivity test for HA and NA genes – separately for the H1N1 (A), H1N2 (B), and H3N2 (C) subtypes.
M – 100 pb DNA ladder (Fermantas, USA). Lanes 1–8 – consecutive 10-fold dilutions of the stock of reference strains.

The optimal concentration of $MgCl_2$ was estimated at 1.2 mM. In the examination of the H1N1 subtypes with an Mg^{2+} concentration equal to or higher than 2 mM, extra unspecific bands were shown between 200 and 300 bp (Fig. 1 A). In the rest of the mPCR tests, the Mg^{2+} concentration has no influence over the specificity and sensitivity of the reaction (Figs 1 B, C).

In the examination of the optimal annealing temperature, the lowest detectable dilution of the reference material was used as the condition in which the amount of artefacts and contamination was decreased. According to the electrophoresis reading, the optimal temperature was estimated as $60^{\circ}C$ for all three tests (Fig. 2).

The sensitivity was adjusted to 10^3 $EID_{50}/0.2$ mL of the reference strains stocks for the H1N1 subtypes, and was visualised in a dilution as low as 10^{-1}. The sensitivity of the mPCR for the H1N2 subtype was visualised in a 10^{-1} dilution of $10^2 EID_{50}$ /0.2 mL. For the H3N2 mPCR test, the sensitivity was observed in a dilution as low as 10^{-3}, which equals 10 $EID_{50}/0.2$ mL (Fig. 3).

The presence of SIV RNA was confirmed directly in 106 samples (36.4%): 90 in nasal swabs (84.9%) and 16 in lungs samples (15.1%). Positive results were received from 20 out of the 30 (66.7%) tested farms. In the molecular subtyping, 19 tested farms were determined as being infected with the H1N1 subtype (95%), and one farm (5%) was infected with the H3N2 virus. Using the conventional virology method, SIV was isolated in 20 cases.

Discussion

The infection of pigs with influenza A viruses is of significant importance to the swine industry and to the epidemiology of human influenza (13). The rapid and sensitive detection of the SIV in clinical specimens is of great necessity in order to avoid the time-consuming and laborious traditional detection of the pathogen. In the majority of cases, the molecular biology based on nucleic acid detection is versatile and applicable. For SIV molecular subtyping, three separate tests were developed. In one PCR test two fragments of the HA and NA genes were decided to be amplified. For the improvement of the analytical reliability of the multiplex PCR, all the parameters involved in the reaction processes, including the temperature, chemicals, and limit of detectability, were considered. In the other cases, more than two primer pairs were used to simultaneously amplification. As was discovered, the most common problem is that some of the primers used in the same reaction tube may interact with each other, thus blocking the reaction (7). Indeed, different primers can significantly alter the sensitivity of the PCR (8, 17). The annealing temperature should be the same for both pairs of primers in one tube in order to eliminate unspecificities. A further problem may be the reliable amount of primers in the reaction. In some conditions, the activity of the primers could be decreased by forming pimer-dimer complexes blocking oligomers. To overcome this problem, very careful selections were made here, including the limit for GC% in oligomers and the simulations of forming pimer–dimers or hairpins. To avoid unspecific polymerase activity in producing DNA products other than the target one, the magnesium concentration was selected as 1.2 mM. In the majority of papers, an Mg^{2+} range estimated as 1.2-1.5 mM was found to give the optimal product yield (3).

In the multiplex RT-PCR that can detect and identified the 16 HA and 9 NA genes directly from the clinical samples, the assays were divided into three groups, with three pairs of primers in each group (2). It was recognised that three pairs of oligomers would be the optimum amount of primers for the temperature conditions in the sensitivity and specificity subtyping. The primer sets were tested with 10-fold serially diluted 10^6 EID_{50}/mL reference influenza A viruses. Amplification could be visualised with a 10^{-5} dilution of each of the reference viruses. The amplification of the multiple DNA targets in one tube differs from the simple parallel merge of the individual PCR (6). In the simulation amplification of the five target influenza virus genes (HA1, H3, H5, N1, N2) the specific bands in the range of the detection limit of 10^{-1} $TCID_{50}$ were equal (14).

For a more complex comparison with one primer pair, the detection limit in the real-time RT-PCR assay proved to be $10^{-0.5}$ EID_{50}/0.2 mL, and $10^{1.5}$ EID_{50}/0.2 mL in the allantoic fluid of virus-infected embryonated chicken eggs and in chicken faeces samples, respectively (9). The other simplex PCR procedure was designed to detect low concentrations of the influenza virus in large volumes of water without the need for costly installations and reagents. The sensitivity of the method was determined using a reverse-genetic H5N1 virus. A concentration as low as 3×10^2 EID_{50}/mL of the initial volume of water was effectively detected (10).

Multiplex RT-PCR with primers for the S1 gene of the infectious bronchitis virus (IBV) and for the fusion protein cleavage site of the Newcastle disease virus (NDV) has a sensitivity of $10^{3.7}$ and 10^3 EID_{50}, respectively (18). Although the multiplex RT-PCR could differentiate the NDV and IBV genomes present in the same sample, there was a slight inhibition of the IBV PCR if a high amount of NDV genome was present in the sample. Thus, to indicate the two different viruses, the PCR procedure was separated for each virus in order to avoid the interaction between the vaccine IBV and NDV. In the other mPCR for the differentiation of the IBV strains using four sets of primers, the sensitivity was established as 10^{-2} EID_{50} (5). This PCR method was used as the second part of the whole IBV identification as nested PCR, following the first step of pre-amplification. The high sensitivity in that case was the consequence of the double step PCR with a nested starter.

In this paper the sensitivity of the evaluated tests was 10^3 EID_{50}/0.2 mL for H1N1 and 10^1 EID_{50}/0.2 mL for H1N2 and H3N2 mPCR. All samples detected as positive in the RT-nested-PCR for the amplification of the conservative gene M1, were confirmed and characterised in the molecular subtyping. The identification and subtyping are important for tracking the prevalent strains in a particular region of the world. Thus, in the other assays detecting the genome of the SIV in clinical samples, the nucleotide information was only taken from the viruses isolated from the particular geographic region (2). Because pigs are often viewed as a "mixing vessel" for both avian and human subtypes of influenza virus, it is of particular interest to know which subtypes are prevalent in the local swine population. Products received from the subtyping PCR assays could be used in epidemiological phylogenesis.

To sum up, the developed and evaluated rapid and specific multiplex PCR assay is an effective additional tool to the current virological methods for the detection and subtyping of the circulating influenza viruses.

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