



COMPARATIVE STUDY OF THE rRT-PCR METHODS FOR RAPID DIAGNOSIS OF INFLUENZA A DISEASE IN PIGS

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Summary

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Swine influenza disease is not included in the National Prophylaxis Programme and the reports about spreading of the virus among pigs are not sufficient. The infection in pigs is usually asymptomatic, but the possible expression of clinical symptoms could result in lower productivity and economic losses. In earlier investigations concerning the spreading of swine influenza viruses (SIV) in Bulgaria, a viral circulation among the swine population was proved. For this reason and because of the high probability of emergence of new strains of unknown infectious potential, it is important to keep current diagnostic readiness in the National Reference Laboratory of Avian Influenza and Newcastle Disease Virus. The goal of the present study was to compare different protocols and combinations of kits and primer/probe sets in order to establish a rRT-PCR protocol for fast SIV diagnosis. To achieve this goal several commercial kits for rRT-PCR detection of SIV were used. After evaluation of the indicators we found that the confidence interval for the Combo M gene protocol in combination with the three used extraction kits ranged between 22.65% and 77.4%, contrasting to the combinations of used in-house amplification protocols with *QIAamp Viral RNA Mini Kit* (QIAGEN, Germany) and *NucleoSpin RNA Virus* (Macherey-Nage, Germany) extraction kits – between 67.05% and 100%. By rRT-PCR, we were able to detect RNA from all swine viruses used in this study. Our results confirmed that the correct detection of swine influenza virus is possible using a protocol different from that of the EU Reference Laboratory.

Key words: Bulgaria, diagnosis, H1N1, influenza, PCR, swine

INTRODUCTION

A brief retrospection of facts shows that the *A/Swine/Iowa/30(H1N1)* strain, isolated by Shope in 1930 (Shope, 1931), is a prototype of a group of viruses, currently known as classical swine H1N1 influenza

viruses, and later replaced by avian-like H1N1 swine strains (Pensaert *et al.*, 1981). After the pandemic in the 1970s, a spread of human H3N2 virus to pigs has been observed (Done & Brown, 1999). In

1984, the reassortment between human H3N2 and avian H1N1 in pigs resulted in a H3N2 reassortant with internal avian gene segments (Castrucci *et al.*, 1993). So far, the infections are asymptomatic, but the H3N2 strain provokes infections with clinical signs. The H1N2 appears in 1990s as a novel triple reassortant derived from human, avian and swine viruses (Brown *et al.*, 1998). The last human pandemic in 2009 was caused by H1N1 influenza virus carrying gene segments derived from swine viruses, originating from North America, Europe and Eurasia, as well as from human and avian influenza viruses (Anonymous, 2009a; Garten *et al.*, 2009).

In response to this pandemic, the European Central Reference Laboratory for Animal Influenza [CRL, VLA Weybridge, UK] created primers and a probe for real-time reverse-transcriptase polymerase chain reaction (rRT-PCR), specifically designed to detect current influenza A viruses known to infect pigs in Europe and North America, and can also detect the pandemic H1N1 2009 [A(H1N1)pdm09] in experimentally infected pigs. The optimised *Combo matrix (M) gene* working protocol was applied successfully in the National Reference Laboratory for Avian Influenza A and Newcastle Disease at the National Diagnostic and Research Veterinary Medical Institute, Sofia even for detection of A(H1N1)pdm09 in human samples (Slavcheva *et al.*, 2011). Afterwards, this protocol was modified by abandoning one of the three *Combo M gene* primers and the modified version was named *Perfect Match* (Anonymous, 2009b).

According to the OIE Terrestrial Manual (OIE, 2010) the detection of IAV in field samples from swine and birds could be performed with several techniques other than the relatively rapid, speci-

fic and sensitive rRT-PCR, which has also a number of disadvantages – probability for obtaining false positive or false negative results due to different reasons and last but not least, the fact that the specific detection of IAV nucleic acid is not 100% equal to infective virus detection. That's why, the “gold standard” for IAV detection and isolation is inoculation of 10–11-day-old chick embryos evaluating the presence of IAV in the allantoic fluid with the haemagglutination (HA) test. Another “gold standard” method is inoculation of cell culture (Madin-Darby canine kidney – MDCK etc.), with observing specific cytopathic effect and further confirmation of IAV presence by haemagglutination test, rRT-PCR, or fluorescent antibody test. For IAVs typing, haemagglutination inhibition (HI) test, fluorescent antibody test, immunohistochemical or enzyme-linked immunosorbent assay (ELISA) tests could be used. Each method has disadvantages – time needed to perform the test, unsatisfactory specificity, efficacy and sensitivity.

Due to the scarce studies of swine influenza in Bulgaria, the provided evidence for circulation of H1N1 and H3N2 viral strains among pig population (Milev *et al.*, 2008) and the current use of a protocol different from the reference one, we aimed to compare 15 different working designs in order to establish the similarities and differences with regard to diagnostic efficacy and sensitivity.

MATERIALS AND METHODS

Viruses

Nine samples from an interlaboratory ring-test for detection of swine influenza viruses from 2008 were used. Three samples were collected from lungs (L), three – from trachea (Tr) and three – from

tonsils (T). Each group of organs contained one of 3 different viral strains – H1N1, H1N2 and H3N2. All samples were tested for presence of IAV within the framework of the test programme and were proven positive.

Viral RNA extraction

Viral RNA from all initial samples was extracted with three different extraction kits following the manufacturers’ protocol. Two of them – *QIAamp Viral RNA Mini Kit* (QIAGEN, Germany) and *NucleoSpin RNA Virus* (Macherey-Nagel, Germany) – were based on the retention of viral RNA after passing the sample through a silica gel membrane filter. The RNA extraction with the third kit – *MagaBio Total RNA Purification Kit* (BioFlux, China) – is based on adsorption of nucleic acid on the surface of magnetic beads and washing using a semi-automated system (GenePure Nucleic Acid Purification System, BIOER TECHNOLOGY, China). In brief, sample sets processed by each of the three kits were marked as Q, MN and BF, respectively.

The initial sample volume when using *QIAamp Viral RNA Mini Kit* was 140 µL,

and elution volume – 80 µL. For *NucleoSpin RNA Virus* the volumes were 150 µL and 50 µL, and with *MagaBio Total RNA Purification Kit* – initial and elution volumes were 100 µL each.

Real-time reverse transcription polymerase chain reaction (rRT-PCR)

To perform this test, five different experimental designs were used with different combinations of amplification kits and primers/probe sets. In all tests, the reverse transcription and amplification was done in one step by *7300 Real-Time PCR System* (Applied Biosystems, USA).

For the first experimental design (Q-C) *QIAGEN One-Step RT-PCR Kit* (QIAGEN, Germany) and the *Combo M gene* protocol of the Central Reference Laboratory – VLA Weybridge, UK (CRL) for detection of the M-gene from the IAV genome through rRT-PCR were used. The sequences of specific primers and probe are listed in Table 1. Thermocycling rRT-PCR conditions were as followed: 50 °C for 30 min, 95 °C for 15 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s, with reading of fluorescence in this step. The final volume of the reaction was

Table 1. Probe and primer sequences used in the comparative study. Bold-typed nucleotides represent the replacements in the Sep2 primer resulting in primer Sep rev-mod. SI – swine influenza; AI – avian influenza

Primer combination	Primer/probe	Sequence
SI – Perfect Match (PM)	Sep 1	AGA TGA GTC TTC TAA CCG AGG TCG
	Sep2 rev-mod	TGC AAA GAC ACT TTC CAG TCT CTG
	SePRO	FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA
SI – Combo M gene (C)	Sep 1	AGA TGA GTC TTC TAA CCG AGG TCG
	Sep2	TGC AAA AAC ATC TTC AAG TCT CTG
	Sep2 rev-mod	TGC AAA GAC ACT TTC CAG TCT CTG
	SePRO	FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA
AI (A)	Sep 1	AGA TGA GTC TTC TAA CCG AGG TCG
	Sep2	TGC AAA AAC ATC TTC AAG TCT CTG
	SePRO	FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA

25 μ L, including 2 μ L template; 5 μ L 5 \times QIAGEN OneStep RT-PCR Buffer, 1.25 μ L MgCl₂ (25 mM), 0.375 μ L ROX Reference Dye, 1 μ L dNTP Mix (10 μ M each dNTP), 1 μ L QIAGEN OneStep RT-PCR Enzyme Mix, 1 μ L RNase Inhibitor (4 U/ μ L, QIAGEN), *SePRO* probe at a final concentration of 250 nM, primer *Sep1* at a final concentrations of 400 nM, and primers *Sep2* and *Sep2 rev-mod*, each at final concentration of 200 nM.

The second design (Q-PM) was based on the updated CRL protocol for detection of M-gene from swine influenza A viral genome through rRT-PCR, the so-called *Perfect match*. It used the same components and parameters for amplification as Q-C except that the primer *Sep2* was excluded and instead, the primer *Sep2 rev-mod* was applied at a final concentration of 400 nM.

The *Combo M gene* primer and probe set was also used in the third experimental variant (K-C) and the reaction was performed with *KAPA PROBE FAST Universal qPCR Kit (KAPA Biosystems, USA)*. Thermocycling rRT-PCR conditions were as followed: 42 °C for 5 min, 95 °C for 5 min, followed by 45 cycles at 95 °C for 3 s and 60 °C for 30 s with reading at this step. The final reaction volume was 20 μ L, consisting of 2 μ L template, 10 μ L KAPA PROBE FAST qPCR Master Mix (2 \times) Universal, 0.4 μ L ROX High/Low, 0.4 μ L KAPA RT Mix (50 \times), and the same concentrations of primers and probe as in the Q-C experimental design.

For the fourth design (K-A) same amplification kit and component's concentrations were used. The *Sep2 rev-mod* primer was excluded from the used M-gene primer and probe set, following the CRL recommendation for detection of avian IAV strains. The final primer and probe concentrations in the mix were 400

nM and 250 nM, respectively. The thermocycling conditions were the same as in the K-C design.

The amplification of the fifth experimental design (F-A) was done by *FLOCKSCREEN AI-4 Real-time PCR Kit (X-OVO, UK)*. The temperature protocol included 50 °C for 20 min, 95 °C for 10 min, 40 cycles at 94 °C for 45 s and 60 °C for 45 s, and reading at this stage. The final reaction volume of 25 μ L included 5 μ L template, 12.5 μ L Master Mix, 2.5 μ L primer and probe mix at final concentration of 400 nM and 150 nM, respectively, and 0.25 μ L Enzyme Mix. The primer and probe sequences were the same as those in the K-A experimental design.

Positive and negative controls

The following positive controls were applied in all reactions: positive template extraction control (PTEC) – *A/swan/Kraimorie/06(H5N1)*; positive template amplification RNA control (PTAC) from the same strain; positive amplification RNA control from the A(H1N1)pdm09 strain. Molecular biology-grade sterile water was used as negative extraction and amplification control. Extraction controls were processed in parallel with the samples according to the respective protocols. Amplification controls were used only at the PCR stage.

Data analysis

The amplification results were analysed by the 7300 PCR system software and expressed in Ct values after manual setting baseline and threshold values for each result.

For each combination of extraction and amplification kit, alternative analysis was performed by evaluation of the percent of PCR-positive samples, the ma-

ximum representation error and the confidence interval range in percent at a level of significance 0.01 (Sepetliev, 1980).

The diagnostic sensitivity (DS) and diagnostic efficacy (DE) were calculated using the following equations:

$$DS, \% = \frac{100 \times TP}{TP + FN}$$

$$DE, \% = \frac{100 \times (TP + TN)}{TP + TN + FP + FN}$$

where TP was the number of true positive samples; TN – the number of the true negative samples; and FP and FN – the number of false positive and false negative samples, respectively.

RESULTS

Ct values

The results from the performed tests in Ct values are presented in Table 2. The H1N1 L sample was out of the detection ability of all used methods. The extraction with *QIAamp Viral RNA Mini Kit* (QIAGEN, Germany) and *NucleoSpin RNA Virus* (Macherey-Nagel, Germany) yielded more stable results compared to those performed by *MagaBio Total RNA Purification Kit* (BioFlux, China). Most samples were detected using F-A, K-C and K-A amplification protocols. The lowest Ct values were obtained using the F-A amplification protocol. Most negative results (undetectable RNA) occurred after using Q-PM and Q-C protocols. The used positive and negative controls demonstrated the expected results.

Alternative analysis

After the evaluation of parameters, it was established that the amplification protocols Q-C and Q-PM for all three extraction kits resulted in confidence interval

ranging between 22.65% and 77.4%, whereas protocols K-C, K-A, F-A and extraction with Qiagen and Macherey-Nagel ranged between 67.05% and 100%.

The results for DS and DE are shown in Table 3.

DISCUSSION

The performed comparative tests on the diagnostic potential of different kits for extraction and amplification of swine influenza virus aimed to determine the optimum set of consumables for our laboratory routine work. The combinations of 3 extraction and 5 amplification protocols resulted in different relative share percentages and different diagnostic sensitivity and efficacy values.

The results indicated that the H1N1 L sample was beyond the detection ability of all used methods. This could be probably due to degradation of the virus and viral RNA after the prolonged storage. In subsequent analyses, this sample was considered negative, in order not to influence the precision of calculations.

Our results showed a low sensitivity of the *MagaBio Total RNA Purification Kit* (BioFlux, China) used for extraction. It could be attributed to the manual addition of the mix containing magnetic beads resulting in their irregular distribution among the samples and thus, inadequate quality of RNA adhesion. The processes in the semi-automated system could also cause poor RNA isolation from our samples. The larger volume of the washing buffer compared to elution volume does not permit the contact of all magnetic beads with the eluent in the elution step, posing a physical obstacle to release the adhered RNA. In previous tests conducted with another commercial kit based on the same principle – *Mag MAX AI/ND Viral*

Table 2. Results from tests (in Ct values) grouped according to used amplification protocols

Extraction	Amplification														
	Q-C			Q-PM			K-C			K-A			F-A		
	Q	MN	BF	Q	MN	BF	Q	MN	BF	Q	MN	BF	Q	MN	BF
H1N1 L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H1N2 L	39.35	-	37.27	37.90	37.69	-	31.92	32.16	34.39	35.54	35.60	38.66	32.59	30.26	30.42
H3N2 L	-	-	-	-	-	-	-	-	-	35.23	34.70	-	36.30	34.64	35.92
H1N1 T	-	-	-	-	-	-	38.79	39.56	-	42.33	-	-	-	-	35.25
H1N2 T	37.82	33.07	35.77	-	-	38.71	28.34	28.10	30.27	25.54	25.39	26.63	24.73	23.17	22.62
H3N2 T	31.13	28.89	29.57	29.98	28.56	30.35	23.80	23.97	26.03	27.92	28.77	29.67	23.40	23.06	21.09
H1N1 Tr	-	-	36.13	39.59	-	-	32.58	34.16	31.14	36.22	42.10	36.09	31.43	32.23	27.19
H1N2 Tr	-	-	-	-	-	35.91	35.79	38.49	-	30.73	31.68	34.34	29.23	29.31	30.85
H3N2 Tr	39.13	-	39.72	34.84	36.88	-	31.20	32.88	36.37	35.72	36.30	40.11	30.10	30.98	31.28
H2O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PTEC H5	31.39	31.37	29.38	33.10	33.67	34.08	26.34	25.14	23.91	24.06	22.41	22.24	14.29	15.11	13.62
PTAC H1 pdm	-	33.52	-	-	31.63	-	-	26.85	-	-	34.80	-	-	32.83	-
PTAC H5	-	34.16	-	-	34.37	-	-	22.02	-	-	19.62	-	-	16.28	-

Table 3. Results for diagnostic sensitivity (DS, %) and diagnostic efficacy (DE, %) for each tested extraction and amplification protocol combination

Extraction kit	Amplification protocol									
	Q-C		Q-PM		K-C		K-A		F-A	
	DS,%	DE,%	DS,%	DE,%	DS,%	DE,%	DS,%	DE,%	DS,%	DE,%
Qiagen	69.23	73.33	69.23	73.33	90.00	91.67	100.00	100.00	90.00	91.67
Macherey-Nagel	60.00	64.71	64.29	68.75	90.00	91.67	90.00	91.67	90.00	91.67
BioFlux	75.00	78.57	64.29	68.75	75.00	78.57	81.81	84.62	100.00	100.00

RNA Isolation kit (Ambion, USA) – we obtained very good results, as the technique allowed obtaining a bigger RNA yield even in the presence of inhibitors (Goujgoulova *et al.*, 2008).

Commercial RNA extraction kits *QIAamp Viral RNA Mini Kit* (QIAGEN, Germany) and *NucleoSpin RNA Virus* (Macherey-Nagel, Germany), used in this comparative study, yielded satisfactory results. The DS and DE percentages with these kits were more consistent unlike the heterogeneous results obtained with the *MagaBio Total RNA Purification Kit*. In field samples, the amount of the virus is often low and therefore, the *MagaBio Total RNA Purification Kit* was assessed as inappropriate for our conditions.

In general, the comparison of amplification results allowed us concluding that the amplification protocols with *Sep1* and *Sep2* primers, used for diagnostic of avian IAV in the practice, were more successful in detecting the swine viral subtypes than those with *Sep2 rev-mod* primer. The PTAC suggested that the typical avian H5N1 strain had lower Ct values after application of all amplification protocols which included *Sep1* and *Sep2* primers rather than those using *Sep2 rev-mod* primer. Opposite to H5N1, the A(H1N1)pdm09 RNA control isolated from a human sample (Slavcheva *et al.*, 2011), yielded lower Ct values after

amplification with *Sep2 rev-mod* primer. The latter is a variant of *Sep2* primer, in which 4 nucleotide bases were changed (Table 1), and which is specific for detection of swine IAV subtypes – the H1N1 avian-like strain, H1N2 and H3N2, and infection in pigs caused by A(H1N1)pdm09. Despite the expectations, the inclusion of this primer in virus detection reactions was not always satisfactory (in over 30% of cases) when a comparison between *Combo M gene* and avian primer set both combined with *KAPA PROBE FAST Universal qPCR Kit* (KAPA Biosystems, USA; K-C and K-A) was made. Yet, both protocols were with comparable DS and DE values.

The *Combo M gene* primers were also applied in the CRL-recommended design for detection of swine influenza A with *QIAGEN One-Step RT-PCR Kit* (QIAGEN, Germany; Q-C). The obtained values did not meet our expectations for best results with this experimental design. This fact motivated us to use the updated CRL protocol for detection of swine influenza A viral strains where the *Sep2* was totally replaced by *Sep2 rev-mod* (Q-PM). Surprisingly, the results were similar to those in the original protocol, with confidence interval limits from 22.65% to 77.4% and unsatisfactory DS and DE percentages regardless to the used extraction kit. Behind these statistical values,

calculated as percent of PCR-positive samples, stand no less important Ct values that could tell approximately whether the result is strongly or weakly positive. Many Ct values obtained from the application of Q-C and Q-PM experimental designs are at the detection limit of the method. This could result in erroneous interpretation of the results as weakly positive or even negative with suspicion for probe degradation, which would be, in fact, a false-negative result impeding the correct diagnosis.

Previous comparative tests on avian IAV performed by us and by other research teams (Slomka *et al.*, 2007) confirmed that the *QIAGEN* kit was the most appropriate one. Our results, although contrary to our anticipations, showed a better sensitivity of amplification protocols used in K-C, K-A and F-A designs compared to those included in the Q-C and Q-PM designs, despite that both have been validated and recommended by the CRL. This could be due to the proportional amount of RNA template in the total volume of the reaction mix, which was 2 out of 25 μ L for Q-C and Q-PM vs 2 out of 20 μ L for K-C and K-A, and 5 out of 25 μ L for F-A.

The *KAPA PROBE FAST Universal qPCR Kit* is a convenient and rapid test, proved to yield sustainable and reproducible results (unpublished data). Here, we can compare the results obtained with it and those obtained with *QIAGEN One-Step RT-PCR Kit* when the primer set was the same, i.e. *Combo M* gene. According to our results, the difference in the obtained confidence intervals: 22.65–77.4 % for Q-C and 67.05–100% for K-C, as well as the DS and DE values, confirmed the superiority of KAPA over QIAGEN.

KAPA PROBE FAST Universal qPCR Kit could be directly compared to

FLOCKSCREEN AI-4 Real-time PCR Kit (X-OVO, UK) in the experimental design where the primers were the same (those for detection of avian influenza A strains). *FLOCKSCREEN AI-4 Real-time PCR Kit* contains enzymes synthesised by *QIAGEN*, specially created on recommendation of OIE and FAO Reference lab in Padova, Italy for detection of M-gene as well as H5, H7 and H9 haemagglutinin subtypes of avian influenza A. It is extremely sensitive as shown from all presented results. With DS and DE of 100% and >91% respectively, and confidence upper limits reaching 92.8%–100%, it provides better relevance of use. Similar results have been obtained also from the application of *KAPA PROBE FAST Universal qPCR Kit* with "avian" primer set (K-A).

CONCLUSIONS

The obtained results suggested that rRT-PCR is a useful tool for detection of swine influenza viruses due to its high sensitivity and short running time.

The precise detection of swine influenza virus is possible by using a protocol, different from that recommended by the OIE Reference Laboratory.

A higher sensitivity and efficacy, with more consistent values were obtained from the extraction of nucleic acids with *QIAamp Viral RNA Mini Kit* (QIAGEN, Germany) and *NucleoSpin RNA Virus* (Macherey-Nagel, Germany).

Using primer pair *Sep1* and *Sep2*, usually applied in practice for diagnosis of avian IAV strains, was more successful for detection of the swine viral subtypes rather than using primer sets with *Sep2 rev-mod* primer.

When comparing the amplification performances, the highest confidence interval was obtained by applying amplifi-

cation kit *KAPA PROBE FAST Universal qPCR Kit* (KAPA Biosystems, USA) and *FLOCKSCREEN AI-4 Real-time PCR Kit* (X-OVO, UK), with primers *Sep1* and *Sep2* and even with the other primer combinations involving *Sep2 rev-mod*.

The highest diagnostic sensitivity and efficacy were obtained with protocol F-A, as well as with K-A and K-C.

The application of the validated and CRL recommended amplification protocols Q-C and Q-PM gave unacceptable results according to all used criteria.

REFERENCES

- Anonymous, 2009a. International Laboratory for Animal Influenza, VLA Weybridge, Pandemic (H1N1) Influenza – Detection in European pigs of H1 gene by H1-118 real time Taqman® RT-PCR, SOP VI.447 Edition 1. <http://www.defra.gov.uk/ahvla-en/files/pub-protocol-si-vi447.pdf> (14 January 2014 date last accessed).
- Anonymous, 2009b. International Reference Laboratory for Swine Influenza – VLA, Waybridge, UK. <http://www.defra.gov.uk/ahvla-en/disease-control/non-notifiable/swine-influenza/> (14 January 2014 date last accessed).
- Brown, I. H., P. A. Harris, J. W. McCauley & D. J. Alexander, 1998. Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in the emergence of an H1N2 virus of novel genotype. *Journal of General Virology*, **79**, 2947–2955.
- Castrucci, M. R., I. Donatelli, L. Sidoli, G. Barigazzi, Y. Kawaoka & R. G. Webster, 1993. Genetic reassortment between avian and human influenza A viruses in Italian pigs. *Virology*, **193**, 503–506.
- Done, S. H. & I. H. Brown, 1999. Swine influenza viruses in Europe. In: *Proceedings from the Allen D. Leman Swine Conference: Track V-Disease*, pp. 255–263.
- Garten, R. J., C. T. Davis, C. A. Russell, B. Shu, S. Lindstrom, A. Balish, W. M. Sessions, X. Xu, E. Skepner, V. Deyde, M. Okomo-Adhiambo, L. Gubareva, J. Barnes, C. B. Smith, S. L. Emery, M. J. Hillman, P. Rivaitler, J. Smagala, M. de Graaf, D. F. Burke, R. A. M. Fouchier, C. Pappas, C. M. Alpuche-Aranda, H. López-Gatell, H. Olivera, I. López, C. A. Myers, D. Faix, P. J. Blair, C. Yu, K. M. Keene, P. D. Dotson Jr., D. Boxrud, A. R. Sambol, S. H. Abid, K. S. George, T. Bannerman, A. L. Moore, D. J. Stringer, P. Blevins, G. J. Demmler-Harrison, M. Ginsberg, P. Kriner, S. Waterman, S. Smole, H. F. Guevara, E. A. Belongia, P. A. Clark, S. T. Beatrice, R. Donis, J. Katz, L. Finelli, C. B. Bridges, M. Shaw, D. B. Jernigan, T. M. Uyeki, D. J. Smith, A. I. Klimov & N. J. Cox, 2009. Antigenic and genetic characteristics of the early isolates of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science*, **325**, 197–201.
- Goujgoulova, G., A. Marinova-Petkova & G. Georgiev, 2008. Demonstration of the significance of the quantity of faecal sample from wild birds for the effect of rRT-PCR inhibitors present in it. In: *Proceedings of International Scientific Conference*, Stara Zagora (BG).
- Milev, D., G. Faldzhiyska, R. Peshev & A. Motovski, 2008. An investigation on spreading of H1N1 Influenza virus among pigs in Bulgaria. In: *Proceedings of the Scientific Conference Tradition and Modernity in Veterinary Medicine*, 189–196 (BG).
- OIE, 2010. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Chapter 2.8.8. Swine influenza, http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.08.08_SWINE_INFLUENZA.pdf (14 January 2014 date last accessed).
- Pensaert, M., K. Ottis, J. Vandeputte, M. M. Kaplan & P. A. Bachmann, 1981. Evidence for the Natural transmission of influenza A virus from wild ducks to swine and its potential importance for man.

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- Bulletin of the World Health Organization*, **59**, 75–78.
- Sepetliev D., 1980, Alternative analysis. In: *Medical Statistics*, 3th edn, Meditsina i fizkultura, Sofia, pp. 69–78. (BG).
- Shope, R. E., 1931. Swine influenza. III. Filtration experiments and aetiology. *Journal of Experimental Medicine*, **54**, 373–380.
- Slavcheva I., G. Goujgoulova, Y. Tumbarski., I. Chenchev & G. Georgiev, 2011. Demonstration of pandemic influenza A virus (H1N1) in samples from patients with polymerase chain reaction in real time (rRT-PCR). In: *Proceedings of the 110 years' NDRVMI Scientific Session*, 7–8, 11, pp. 286–291 (BG).
- Slomka, M. J., V. J. Coward, J. Banks, B. Z. Londt, I. H. Brown, J. Voermans, G. Koch, K. J. Handeberg, P. H. Jorgensen, M. Cherbonnel-Pansart, V. Jestin, G. Cattoli, I. Capua, A. Ejdersund, P. Thoren & G. Czifra, 2007. Identification of sensitive and specific avian influenza polymerase chain reaction methods through blind ring trials organized in the European Union. *Avian Diseases*, **51**, 227–234.
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