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A multiplex real-time PCR assay for detection of oseltamivir-resistant strains of influenza virus

Research Article

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Abstract: Influenza is a contagious disease of humans and animals caused by viruses belonging to the *Orthomyxoviridae* family. The influenza A virus genome consists of negative sense, single-stranded, segmented RNA. Influenza viruses are classified into subtypes based on two surface antigens known as hemagglutinin (H) and neuraminidase (N). The main problem with influenza A viruses infecting humans is drug resistance, which is caused by antigenic changes. A few antiviral drugs are available, but the most popular is the neuraminidase inhibitor – oseltamivir. The resistance against this drug has probably developed through antigenic drift by a point mutation in one amino acid at position 275 (H275Y). In order to prevent a possible influenza pandemic it is necessary to develop fast diagnostic tests. The aim of this project was to develop a new test for detection of influenza A virus and determination of oseltamivir resistance/sensitivity in humans. Detection and differentiation of oseltamivir resistance/sensitivity of influenza A virus was based on real-time PCR. This test contains two TaqMan probes, which work at different wavelengths. Application of techniques like multiplex real-time PCR has greatly enhanced the capability for surveillance and characterization of influenza viruses. After its potential validation, this test can be used for diagnosis before treatment.

Keywords: *Influenza • Detection • Oseltamivir • H275Y • Real-time PCR*

1. Introduction

Each year influenza viruses cause considerable morbidity and mortality all over the world. Only in the US, more than 200 000 people are hospitalized and approximately 36 000 die from influenza-related diseases annually [1]. The best defense against the virus is vaccination, which unfortunately is limited by difficulties in the selection of appropriate antigens from the circulating strains of the virus. Additionally, the level of immunity in the population is limited if the vaccine coverage is less than 100%. Due to these reasons, influenza virus can easily spread in the human population.

Therefore, antiviral drug therapy is necessary for fighting against this pathogen. The first generation anti-influenza drugs – ion channel blockers (amantadine and ribavirine) – had to be removed from clinical use due to widespread resistance. It is estimated that 80% of isolated viruses are immune to these drugs [2]. The second generation drugs – neuraminidase inhibitors – became the only effective antiviral drugs. Currently, there are two drugs approved: oseltamivir (Tamiflu, Roche) and zanamivir (Relenza, GlaxoSmithKline). In order to be effective, they have to be administered within the first 48 hours after infection [3].

There are two known mechanism that contribute to neuraminidase (NA) resistance of influenza viruses [4]. One mechanism involves reduction of binding efficiency of viral hemagglutinin to its receptor. Another is associated with substitution of amino acids around

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the active site of NA [5]. The first cases of oseltamivir-resistant strains of influenza A/H1N1 were reported in 2008 in Europe [6]. Analysis of the drug-resistant strains revealed that all the viruses carried the same C->T transition mutation in the neuraminidase gene in position 823. This results in a histidine-tyrosine change at amino acid position 275 (H275Y or H274Y, according to the universal N2 numbering) [7].

Detection of a resistant virus was usually performed by a phenotypic assay such as the neuraminidase inhibition assay [8], or by sequencing of the viral genome [9]. However, these methods are labor-intensive and time consuming, and require specialized equipment. Moreover, the sensitivity of sequencing assays is often too low for samples with low viral level.

In this study, we present a one-tube multiplex real-time PCR assay for detection and differentiation of oseltamivir resistant/sensitive strains of influenza virus. There are several molecular methods based on PCR and real-time PCR analysis, but none of them offers a multiplex real-time PCR assay using only two standard TaqMan probes [10-13].

2. Experimental Procedures

2.1 RNA extraction and cDNA synthesis

RNA was extracted from media after growth of the virus in MDCK culture and/or oral swabs using the Total RNA Mini Kit (A&A Biotechnology, Poland) protocol. Synthesis of the first strand of cDNA was carried out according to the procedure described for the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). The reaction mix containing 5 µl viral RNA (10-30 ng μL^{-1}), random hexamer primers (2 µl, 0.6 mM L^{-1}), and water (4.5 µl) was incubated for 10 min at 65ºC. Subsequently, reaction buffer, RNase inhibitor, dNTPs, DTT, and reverse transcriptase were added. The final mixture contained 1x reaction buffer, 20 U of RNase inhibitor, 60 µM L^{-1} of random hexamer primers, 1 mM L^{-1} of each dNTP, 5 mM L^{-1} of DTT, and 10 U of reverse transcriptase. It was then incubated for 10 min at 29°C followed by a 60-min incubation at 48°C and a 5-min incubation at 85ºC, and then cooled on ice.

2.2 Site-directed mutagenesis

To modify the neuraminidase gene, site-directed mutagenesis was applied, by using QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Inc. Santa Clara, CA, USA)

As a template for mutagenesis, pPolI-NA-mex4486 plasmid (kindly provided by Wendy S. Barclay and Lorian C. S. Hartgroves from the Department of Virology, Imperial College of London) was used. The primers shown in Table 1 were used to generate the mutation at position 823 of the neuraminidase gene. This plasmid was used as a control for oseltamivir-resistant strains.

At the first stage of reaction, PCR amplification was performed using the following mixture: both primers (1.25 µl of MutNA_T_rev (100 ng μL^{-1}) and 1.25 µl of Mut NA_T_for (100 ng μL^{-1})), 5 µl of reaction buffer, 1 µl of dNTPs mixture (10 mM L^{-1}), 1.5 µl of QuikSolution reagent, 1 µl of *Pfu Turbo* polymerase, and 38 µl of dH_20. The mixture was initially denatured at 95°C for 2 min, followed by 18 cycles comprising 20 s of denaturation at 95°C, 10 s of annealing at 60°C, and 220 s of extension at 68°C, and concluded by 5 min of final extension at 68°C.

The next stage of reaction was digestion of the PCR products with *DpnI* enzyme. 2 µl of enzyme was added to the PCR mixture, mixed, and incubated at 37°C. After 2 hours of incubation, 45 µl of XL-Gold ultracompetent cells with 2 µl of β-mercaptoethanol was transformed

Primer/probe	Sequence (5'→3')	Position	Application
MutNAfor	GAATGCCCCTAATTATTACTATGAGGAATGCTCCTG	807-842	Mutagenesis
MutNArev	CAGGAGCATTCCTCATAGTAATAATTAGGGGCATTC	807-842	
IV-M-For	AGATGAGTCTTCTAACCGAGGTCG	25-48	
IV-M-Rev	TGCAAAAACATCTTCAAGTCTCTG	101-124	Detection of M gene [15]
IV-M-Probe	*FAM*-TCAGGCCCCCTCAAAGCCGA- *BBQ1*	64-83	
IV-NA-For	ATGACCGATGGACCAAGTA	724-742	
IV-NA-Rev	AGCCATGCCAGTTATCCCT	877-895	Detection of oseltamivir sens./res.
IV-NA-Probe	LC610-CCTAATTATCACTATGAGGAATGCTCCTGTTATC-BBQ2	814-847	

Table 1. Primer and probe sequences used in the assay.

BBQ – Blackberry Quencher, FAM - 6 – carboxyfluorescein, LC610 – LightCycler® Red 610

by using the digested mixture. After overnight incubation at 37°C, a colony from the Petri dish was taken and proliferated in a liquid culture overnight.

Next, the plasmid carrying the mutation was isolated using Plasmid Mini kit (A&A Biotechnology, Gdynia, Poland). Correctness of the purified plasmid sequence was confirmed by Sanger sequencing.

2.3 Primer and probe design, and multiplex real-time PCR

In silico analyses were performed with Geneious software (http://www.geneious.com/) using sequences available in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Primers and probes used in the assay are listed in Table 1. BLAST sequence analyses were also performed to confirm the specificity and selectivity of the designed primers and probes.

Multiplex real-time PCR was performed using LightCycler 2.0™ (Roche Diagnostics, Mannheim, Germany). 20 µl of the final mixture contained 17 µl of the reaction mixture: 1x LC TaqMan Master (Roche Diagnostics, Mannheim, Germany), 0.25 µM L^{-1} of each primer, 0.1 µM L^{-1} of each TaqMan probe, and 3 µl of cDNA. A negative control lacking cDNA was also included. The samples were initially denatured at 95ºC for 10 min, followed by 45 cycles composed of 95ºC (denaturation) for 10 s, 56ºC (annealing) for 15 s, and 72ºC (extension) for 7 s. Fluorescence was measured at two wavelengths: 530 nm for IV-M probe (FAM) and 610 nm for IV-NA probe (LC610).

2.4 Sensitivity and specificity of the assay

The detection limit was determined using a target copy number of fragments of influenza virus M gene (pPolI-M-E195) and NA gene (pPolI-NA-mex4486). Plasmid DNA was extracted from bacteria by using Plasmid Mini Kit (A&A Biotechnology, Gdynia, Poland). The concentration of plasmid DNA was determined by spectrophotometry. DNA copy number was calculated using the formula described by Ke *et al.* [14]. The plasmid was serially diluted 10-fold to serve as a standard for determining the sensitivity of the real-time PCR assay. cDNA was added to the reaction mixture as a template.

The specificity of the newly developed assay was investigated using the RNA extracted from other viruses including LaSota – reference AF077761, APMV-1/chicken/Poland/111/90, APMV-1/chicken/Poland/Radom/70, Italy/2736/00 – reference AY562989, Coronavirus 165/08 5-8, Rotavirus G036/10 1-5, Gumboro virus 131/92, APMV – 2, APMV – 3, APMV – 4, kindly provided by Department of Poultry Diseases, National Veterinary Research Institute, Pulawy, Poland.

3. Results

3.1 Interpretation of the multiplex real-time PCR assay

The results of influenza detection and differentiation of oseltamivir sensitivity/resistance based on multiplex real-time PCR was obtained by using two TaqMan probes labeled with FAM and LC610 fluorescent dyes, respectively. The results of analysis are shown in Figure 1.

General detection of the presence of influenza virus in the sample was based on detection of the M gene using the 530 nm channel of LightCycler. The analysis of sensitive/resistant strains was performed using the 610 nm channel. Positive results in this channel indicate oseltamivir sensitivity of an influenza strain.

3.2 Detection limit

The detection limit of the assay was assessed by analyzing serial dilutions of plasmids carrying fragments of the M and NA genes of the influenza virus. For the multiplex real-time PCR assay, the detection limits were about 400 copies of plasmid DNA per reaction for both genes. This result is similar to those of previously described methods.

3.3 Specificity and selectivity of the assay

The specificity of the new assay was investigated by analysing 10 heterologous pathogens. No amplification products were detected when templates originating from those ten other, unrelated viruses were used.

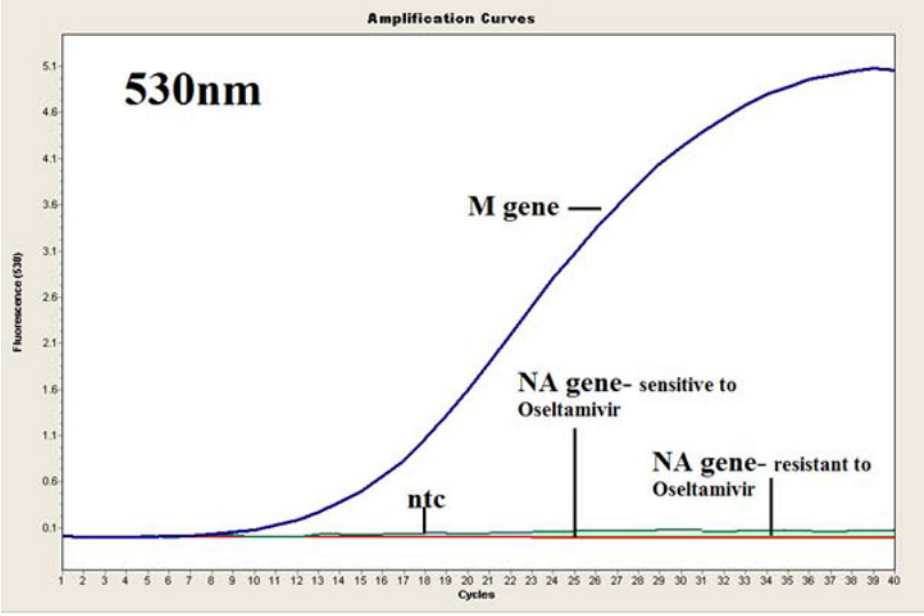
Selectivity of the assay was confirmed by analysis of avian and human strains of influenza. All influenza strains are detected using IV-M-For and IV-M-Rev primers and IV-M-Probe. However, only human strains (H1N1) may by analysed by NA primers and probe.

4. Discussion

As the first generation drugs like amantadine or rimantidine are no longer effective, due to influenza virus resistance, the second generation drugs are the only weapon for fighting this pathogen. Unfortunately, some strains have gained resistance against these drugs as well.

The resistance to oseltamivir becomes a serious problem for potential treatment of people during an epidemic or pandemic. Diagnostics of sensitivity/resistance to neuraminidase inhibitors is very important. It is also very important to reduce the time of analysis by using the most recent molecular methods, in order to apply antiviral drugs in time.

A



B

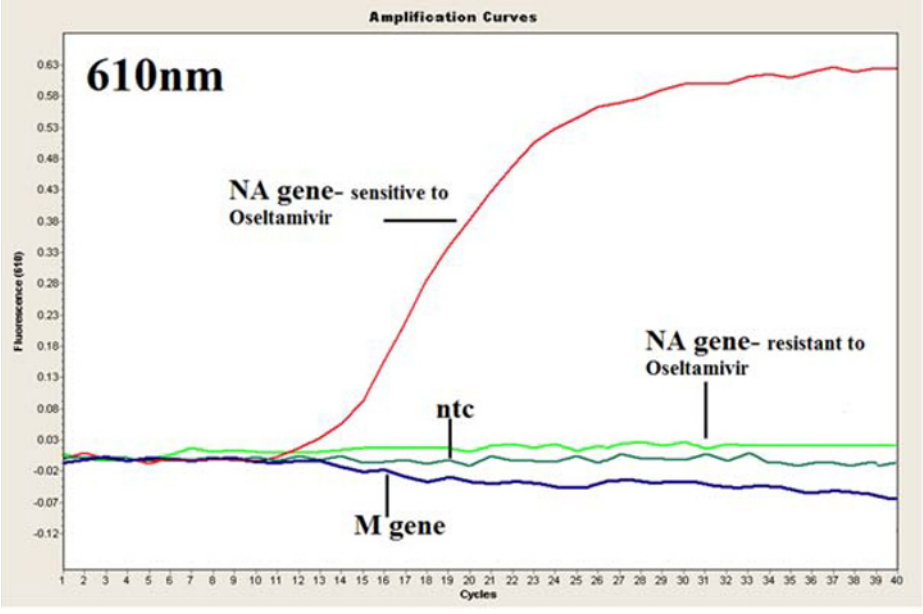


Figure 1. Multiplex real-time PCR analysis for detection of influenza virus and differentiation of oseltamivir sensitive/resistant strains. A – Amplification curve at 530 nm – used for detection of M gene of influenza virus. B – Amplification curve at 610 nm – used for detection of influenza virus NA gene sensitive to oseltamivir. ntc – no template control

In this study, we present a one-tube multiplex real-time PCR assay for detection of influenza virus and differentiation of resistant/sensitive oseltamivir strains. Influenza virus was detected using the 530 nm channel of LightCycler by analysis of the viral M gene. The M gene is the most conserved part of the influenza virus genome and was used for detection in a previous study [15-17]. For differentiation of oseltamivir sensitive/resistant strains we used NA gene analysis. A single mutation at the amino acid position 275 (H275Y or H274Y, according to the universal N2 numbering) causes resistance to oseltamivir. For detection of the NA gene we used a probe labeled with LC610, and for the probe detection the 610 nm channel of LightCycler was used.

In previously described tests, the authors used LNA probes [10], High-Resolution Melting [11], the Cycling probe method [18], or two probes for detection of sensitive and resistant strains [13,19-22]. There are only a few multiplex assays for detection of influenza virus and determination of oseltamivir sensitivity/resistance, and they are based on conventional PCR [12], or need three probes in the assay [23].

In conclusion, we describe a test which allows detection of influenza virus and differentiation of oseltamivir sensitive and resistant strains in one reaction, and in a relatively short time. Using conserved fragments of M gene and a variable fragment of NA makes this method very universal for all serotypes of influenza virus. The sensitivity of the assay was several hundred copies of virus for both general detection and differentiation of oseltamivir resistant and sensitive strains. These results are somewhat worse compared to those of a previous study but still good enough to be useful for diagnostics. In our opinion, the test may serve as the first step of influenza diagnostics for making decisions about further treatment of patients.

Additionally, the presented test can serve as a useful tool for surveillance of virus infections and monitoring of drug resistance among the viruses in the environment.

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Conflict of interest

The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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