POLYMERASE CHAIN REACTION (PCR) - AN EFFICIENT TOOL FOR DIAGNOSIS OF INFLUENZA AND ACUTE RESPIRATORY ILLNESSES DURING INFLUENZA SEASON

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SUMMARY

Many severe diseases of the respiratory tract lead to hospitalisation. These diseases are often caused by viral infections and may cause increased mortality. The most common viral pathogens involved in these cases, which are also associated with significant morbidity and mortality during the influenza seasons are influenza viruses. Rapid differential diagnosis of influenza viruses is therefore of great importance. Classical diagnosis of these viruses involves virus cultures. Of the rapid diagnostic methodologies which have been developed are RT-PCR, multiplex PCR, real-time PCR. In the present study we have monitored clinical samples from patients of different age groups from selected regions in Slovakia and compared the effectiveness of the classical and molecular biological diagnostic methods. The molecular biological methods proved to be rapid, accurate and effective. Application of these techniques in diagnosis of the respiratory illnesses should help in the prevention, therapy and disease control.

Key words: influenza viruses, diagnostic choices, cultivation, RT-PCR, real-time PCR, effectiveness of diagnosis

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INTRODUCTION

Human influenza is a highly contagious acute respiratory tract disease, which can cause severe morbidity and mortality, particularly in elderly or immuno-compromised patients (1). Three distinct immunological types of influenza viruses have been described on the basis of antigenic differences in the matrix (M) protein and the nucleoprotein. Beside other differences influenza viruses A and B are asociated with seasonal morbidity and mortality, while influenza C causes mild upper respiratory tract infections in children and adolescents (2, 3). Influenza virus A is further classified into different subtypes according to antigenic and genetic differences in its surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Fifteen HA subtypes and 9 NA subtypes have been identified to date. Until recently, only H1N1, H2N2, and H3N2 subtypes have been associated with widespread epidemics in humans. Influenza virus A subtype H1N2 emerged and caused respiratory infections in patients from Israel and other countries during the 2001–2002 influenza season (4, 5, 6). The antigenic variability of influenza A arises as a result of antigenic shift and antigenic drift. Rate of mutation in the influenza virus genome is very high (7, 8).

Influenza infections occur in well expressed seasonal patterns. They are a cause of serious worry as serious medical conditions and complications such as debilitating febrile illness lasting for 1–2 weeks can occur in healthy young adults, in patients with pre-existing respiratory or cardio-vascular disease, and in the frail elderly persons. One characteristic of seasonal influenza activity is that it leads to excess or hidden deaths. These deaths are not usually attributed to influenza virus infection but caused either by viral or bacterial pneumonia, secondary to influenza infection (7).

Rapid diagnosis of influenza infections is important for surveillance, prevention and therapy. Traditional method (gold diagnostic standard) for diagnosis of influenza virus infections, is egg inoculation where the virus replicates in the amniotic fluid. Egg inoculation is not practical in all diagnostic laboratories; the method is still used for producing current vaccines, because egg cultivation remains the best method of quickly generating very high titres of virus (22). In diagnostic laboratories, usually the tissue cultures isolation methods are applied. The commonly used cell lines are primary rhesus monkey kidney, cynomolgus monkey kidney, or Madin-Darby canine kidney (MDCK). Virus is detected by examining the cells for cytopathic effect (CPE) and

expression of viral hemagglutinin on the surface of infected cells. Viral hemagglutinin is detected by adding a suspension of guinea pig erythrocytes to the culture and examining it for adsorption of the erythrocytes to the surface of the cells in culture (hemadsorption). The advantage of virus culturing is its sensitivity, but the main disadvantage is that the CPE or HA expression usually takes 2–3 days to develop, interpretation of results may take as long as 7 to 10 days (4, 22–24). Although traditional culturing method is one of the most sensitive methods for the detection of virus, it often does not provide results in a time frame that is clinically relevant. The antiviral therapy is only effective when given in the first 48 hours after the onset of symptoms, for this reason a shell vial culture method was developed. The rapid culture methods have been shown to be 60% to 100% sensitive in detecting influenza virus relative to traditional culture methods (25–27). The main drawback of this method is the increased level of expertise required for performing and interpreting these types of cultures.

Detecting increase in antibody titers in paired acute and convalescent sera (collected 3 to 4 weeks apart) can be a useful tool for diagnosing infections, but this method is usually applied only when other attempts of diagnosis fail. Traditional serologically assays are complement fixation test and hemagglutinin inhibition. Serological assays for diagnostic or epidemiologic purposes do not fit into the routine of most clinical laboratories but these assays are often reserved for large reference centres or public health laboratories (22).

Microscopic techniques apply fluorescent labeled antibodies for detection of the virus on slide specimens. Although this method is useful, it is less sensitive (22, 28–32).

Viral antigen can be also detected in clinical specimens using commercial enzyme immunoassay devices. The best known are Directigen® Flu A (Directigen® Flu A+B) (Becton-Dickinson, Cockeysville, Maryland), FLU OIA® (Biostar, Boulder, Colorado) and ZstatFlu® (ZymeTx, Oklahoma City, Oklahoma) (22). These devices vary regarding the time required for performance of the test (15–45 minutes), the virus types detected (influenza A only or both influenza A and B), test format structure, and the specimens recommended for testing (22). Directigen® Flu A from Becton-Dickinson, (Cockeysville, Maryland) is the most widely used antigen detection device and also the most comprehensively evaluated in the literature (22). Directigen® reports sensitivities ranging from 41% to 100% and specificities of 71% to 100%, depending on the type of specimen collected, conditions during sample collection, and patient populations studied (22, 28, 30, 33–36).

Rapid and sensitive molecular diagnostic techniques for the detection of influenza viruses have been developed and evaluated. Conventional RT-PCR is one of the best-known methods (4, 37, 38, 39). In the so-called „multiplex“ format, PCR assays have been designed to amplify more than one respiratory viral target in the same PCR test (31, 40–45).

Real-time RT-PCRs and multiplex real-time PCRs for the detection of influenza viruses in clinical samples have recently been described (44, 46–49). Commercial real-time PCR instruments that have recently become available include the ABI PRISM 5700 a 7700 (Applied Biosystems, Foster City, CA), iCycler (BioRad, Hercules, CA), LightCycler instrument (Roche Molecular Biochemicals, Indianapolis, IN), SmartCycler (Cepheid, Sunnyvale, CA), MX4000 (Strategene, La Jolla, CA) a Roto-Gene (Corbett Research, Sydney, Australia) (50). Probe detection formats, which have been most frequently adapted to real-time instruments, include TaqMan (Roche Diagnostics Corporation), Molecular beacons and FRET (Fluorescent Resonance Energy Transfer, Roche Molecular Biochemicals) (50).

Symptoms and signs of influenza may be similar to other types of respiratory illnesses, prompt diagnosis of influenza infection would therefore facilitate effective patient management, public health and vaccination programs as well as appropriate use of antiviral therapy (9, 18–21). Considering the importance of rapid diagnosis, we have compared the classical viral culture method and modern molecular methods such as RT-PCR and real-time PCR for diagnosing influenza viruses and other infectious agents during the influenza season in patients with respiratory illnesses.

MATERIAL AND METHODS

Sample Collection

Clinical samples such as nasopharyngeal swabs and post-mortem material, or other biological material from patients were taken by sentinel and nonsentinel physicians (Children Faculty Hospital Banská Bystrica, Department of Pneumology; Health Care Surveillance Authority, Medico-Judicial and Pathological-anatomical Workplace Banská Bystrica) from central Slovakia region (region of Banská Bystrica and region of Žilina). Patients included both adult and pediatric patients from sentinel and nonsentinel physicians from central Slovakia region. Nasopharyngeal swabs were collected and placed into containers with viral transport medium (Medium 199 with 0,5% BSA and antibiotics Penicillin G, Streptomycin and Amphotericin) (WHO 2002) supplied by the laboratory.

These samples were then delivered for analysis to Regional Authority of Public Health Banská Bystrica, Division of Medical Microbiology, Departments of Molecular Biology and Medical Virology. We monitored the presence of influenza viruses by cultivation and PCR methods. Furthermore, differential diagnosis of influenza virus employing PCR and real-time PCR methods was done.

Viral Culture

Viral cultures were carried out in accordance with standard virological techniques and WHO protocol.

Tissue culture isolations: Specimens were inoculated into culture monolayers of Madin-Darby canine kidney (MDCK) cells and into 8–10 days old chicken embryos and passaged at least three times in these cells or chicken embryos before reporting inability to recover virus from the specimen.

MDCK cells were maintained in D-MEM medium with L-glutamine containing 0,2% bovine serum albumin, HEPES buffer, 10% fetal bovine serum and antibiotics at 37 °C in 5% CO_2. Cells were replaced after a maximum of 15 sequential passages. Infected monolayers of cells were maintained in the same D-MEM medium, but with TPCK-trypsin and without fetal bovine serum at 34 °C in 5% CO_2. Cells were monitored for cytopathic effect (CPE) and observed daily under a light microscope at 40x magnification. Passages were maintained at least 7 days and stopped if 3+ or 4+ CPE was observed. Every second day

a hemagglutination test was performed. We used three kinds of red blood cells – chicken (0,5% suspension), guinea pig and human type 0 (both 0,75% suspension).

Inoculated 8–10 day´s embryonated chicken embryos were incubated at 34 °C for 2–3 days. A hemagglutination test with three kinds of red blood cells was performed with harvested fluids. Uninoculated residual specimens were stored at –70 °C.

Isolation of Viral RNA from Samples

Viral RNA was isolated from clinical samples using the commercial kit (Viral Nucleic Acid Isolation Kit, Roche Diagnostics 2002), the mannufacturer´s protocol was followed. To each series of nucleic acid isolation a sample of deionized water was integrated. This sample was used as a negative control for nucleic acid isolation.

Detection of Influenza Viral RNA Using PCR

For detection of influenza RNA in biological material samples we used a conventional RT-PCR method. Diagnostic sets GenePak® RNA PCR TEST InfluenzaA and GenePak® RNA PCR TEST InfluenzaB from GENTECH (Moscow) were used. Reactions of reverse transcription and PCR were made separately under different conditions of reaction (GENTECH, 2005). Reverse transcription conditions: 25 °C/10 minutes, 40 °C/30 minutes, 50 °C/10 minutes. After the addition of STOP solution: incubation at 80 °C/15 minutes.

PCR conditions: Denaturation: 95 °C/2 minutes. Amplification: 45 cycles 95 °C/20 seconds, 58 °C/20 seconds, 74 °C/ 40 seconds; 74 °C/2 minutes. We used Hybaid PCR express thermal cycler© Hybaid Limited for RT-PCR.

Control of reaction: At the same time and under the same conditions, the control of procedure of RT-PCR was inspected using the positive (RNA of influenza A or B virus) and the negative control (deionized water) of reaction, which are included in the Diagnostic Kit.

Electrophoresis: After the completion of amplification in thermocycler, the PCR products were analysed with UV light after electrophoresis in agarose gel (90 V/1 hour; 120 V/2 hours).

Differential diagnosis of influenza: Apart from influenza viruses diagnosis we also tried a differential diagnosis of influenza viruses. Presence of other viral agents such as parainfluenza virus, RSV-respiratory syncytial virus, adenovirus, human metapneumovirus, and bacterial respiratory agents such as *Streptococcus pneumoniae*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Pneumocystis carinii*, *Bordetella pertussis* and *Bordetella parapertussis* was inspected.

Conventional PCR was applied for the diagnosis of adenovirus, *Streptococcus pneumoniae, Chlamydia pneumoniae, Mycoplasma pneumoniae*, *Pneumocystis carinii*), RT-PCR (parainfluenza virus, RSV, human metapneumovirus and the real-time PCR for the diagnosis of *Bordetella pertussis* and *Bordetella parapertussis*.

External quality assessment: The assessment was done at the Departments of Medical Virology and Molecular Biology, Division of Medical Microbiology, Regional Authority of Public Health, execute laboratory practice in accordance with STN ISO EN/IEC 17 025:2005. This work place attends on international quality assurance.

In addition during October 2007 Regional Authority of Public Health, Division of Medical Microbiology, Departments of Medical Virology and Molecular Biology, attended on external quality assurance last time. This quality control assurance was organized by National Reference Centre for Influenza laboratory in Bratislava. Both departments were 100% successful.

The diagnostic sets [GenePak® RNA PCR TEST InfluenzaA and GenePak® RNA PCR TEST InfluenzaB, GENTECH (Moscow)] were tested using reference materials provided by National Reference Centre for Influenza laboratory.

RESULTS

In 2005/2006 influenza season 229 biological samples were examined in department of Medical Virology, Division of Medical Microbiology, Regional Authority of Public Health Banská Bystrica. Virus isolation was made by cultivation on MDCK tissue cultures and chicken embryos. The presence of influenza virus was detected in 13 cases. All strains were detected on MDCK tissue cultures and one of them was also detected on chicken embryo. All 13 positive samples were delivered to the laboratory of the National Reference Centre for Influenza (Authority of Public Health of Slovak republic in Bratislava) for typing. The strains were typed as influenza B/Malaysia/2506/2004-like (12 samples) or like influenza A/H3N2/California/07/04-like (1 sample).

In 2006/2007 influenza season 176 nasopharyngeal swabs were examined in Department of Medical Virology, Division of Medical Microbiology, Regional Authority of Public Health Banská Bystrica. The presence of influenza virus in examined samples was detected in 3 cases. All strains were detected on MDCK tissue cultures and two of them were also detected on chicken embryos. All 3 positive samples were delivered to the National Reference Centre for Influenza laboratory in Bratislava for typing. The strains were typed as influenza A/Wisconsin/67/2005-like. The results from 2005/2006 and 2006/2007 seasons are summarized in Tables 1 and 2.

Table 1. *Diagnosis of influenza in 2005/2006 influenza season by tissue culture and embryo cultivation methods*

01. 09. 2005 – 31. 05. 2006		
Examination	Cultivation on MDCK tissue cultures	Cultivation on chicken embryos
All samples	229	229
Positive samples	13	1

Table 2. *Diagnosis of influenza in 2006/2007 influenza season by tissue culture and embryo cultivation methods*

01. 09. 2006 – 31. 05. 2007		
Examination	Cultivation on MDCK tissue cultures	Cultivation on chicken embryos
All samples	176	176
Positive samples	3	2

In the 2005/2006 influenza season 78 biological materials were examined for the presence of different infectious agents causing respiratory tract infections by RT-PCR in department of Molecular Biology, Division of Medical Microbiology, Regional Authority of Public Health Banská Bystrica. The presence of influenza A virus was tested in 74 samples and the presence of influenza B virus was tested in all 78 samples. The presence of influenza A virus was detected in 3 cases. The presence of influenza B virus was detected in 7 cases. Differential diagnosis of influenza was performed in the same time, too. Samples were tested for the presence of influenza A-H5, H7, *Pneumocystis carinii*, *Mycoplasma pneumoniae*, adenovirus, parainfluenzavirus, respiratory syncytial virus (RSV), *Bordetella pertussis* and *Bordetella parapertussis*. Results of diagnosis and differential diagnosis of influenza viruses, in 2005/2006 influenza season, by molecular biology methods are summarized in Table 3.

In 2006/2007 influenza season 99 biological samples were examined for the presence of respiratory tract infection agents by RT-PCR in department of Molecular Biology, Division of Medical Microbiology, Regional Authority of Public Health Banská Bystrica. The presence of influenza A virus was tested in 171 samples and the presence of influenza B virus was tested in 157 samples. The presence of influenza A virus was detected in 13 cases. The presence of influenza B virus was detected in 1 case. Differential diagnosis of influenza was performed in the same time, too. The samples were tested for the presence of influenza A-H5, H7, *Pneumocystis carinii*, *Mycoplasma pneumoniae*, adenovirus, parainfluenzavirus, respiratory syncytial virus (RSV), *Bordetella pertussis*, *Bordetella parapertussis*, *Chlamydia pneumoniae*, human metapneumovirus (HMPV), *Streptococcus pneumoniae*. Results of diagnosis and differential diagnosis of influenza viruses, in 2006/2007 influenza season, by molecular biological methods are summarized in Table 4.

In 2005/2006 and 2006/2007 influenza seasons in total 217 biological samples were examined both by cultivation on MDCK tissue cultures and by RT-PCR. Four samples were positive by cultivation on MDCK tissue cultures. At the National Reference Centre for Influenza these cases were typed as subtype of influenza A/Wisconsin/67/2005-like in one case, and as influenza B/Malaysia/2506/2004-like in three cases. By molecular biological method, RT-PCR, the presence of influenza virus was detected in 20 cases. In fourteen positive cases influenza A strains and in 6 influenza B virus were detected. Results of parallel testing are summarized in Table 5.

DISCUSSION AND CONCLUSIONS

Surveillance for influenza viruses in population is important for providing information concerning the presence of the influenza virus subtypes circulating in the population. This information is useful in vaccine development and for timely beginning of antiviral therapy, too (4, 9, 10).

Vaccination is the best choice for prevention of the illness. The targeted population includes high risk patients due to comorbidity, all patients aged over 65 years, children and immuno-compromised individuals (11).

Antiviral drugs can be used for prophylaxis of unvaccinated persons who are exposed to influenza, and for treatment of both vaccinated and unvaccinated patients who develop typical symptoms. Prophylaxis with an antiviral drug may be useful for the unvaccinated people or if circulating strains prove to be different from vaccine strains (reviewed in 12, 13, 14, 17).

We tested in total 217 biological samples during the 2005/2006 and 2006/2007 influenza seasons. Samples were processed and virus culture methods, RT-PCR and real-time PCR were used for

Table 3. *Differential diagnosis of influenza during 2005/2006 influenza season using molecular-biology methods*

01. 09. 2005 – 31. 05. 2006									
Examination	IA*	IB*	A-H5, H7	PC*	MP*	AV*	PI*	RSV*	BP+BPara*
All samples	74	78	7	20	57	50	54	64	21
Positive samples	3	7	0	2	0	0	0	1	0

*For explanation see Table 4.

Table 4. *Differential diagnosis of influenza during 2006/2007 influenza season using molecular-biological methods*

01. 09. 2006 – 31. 05. 2007												
Examination	IA	IB	A-H5, H7	PC	MP	AV*	PI*	RSV	BP+BPara	CHP*	HM*PV*	SP*
All samples	99	85	1	58	67	64	58	74	61	56	22	80
Positive samples	13	1	0	1	0	0	0	1	1	0	0	61

*Abbreviations used:

IA:Influenza A
IB: Influenza B
A- H5, H7: Influenza A, subtypes H5, H7
PC: *Pneumocystis carinii*
MP: *Mycoplasma pneumoniae*
PI: Parainfluenza
BP: *Bordetella pertussis*
RSV: Respiration syncytial virus
CHP: *Chlamydia pneumoniae*
HMPV: Human metapneumovírus
SP: *Sterptococcus pneumoniae*
AV: Adenovirus
Bpara: *Bordetella parapertussis*

Table 5. *Comparison of classical cultivation method and molecular-biological methods for diagnosis of influenza in 2005/2006 and 2006/2007 influenza seasons*

	Influenza season 2005/2006			Influenza season 2006/2007		
	Cultivation	RT-PCR		Cultivation	RT-PCR	
		Influenza A	Influenza B		Influenza A	Influenza B
All samples	64	64	64	153	153	153
Positive samples	1	3	6	3	11	0

Total		
	Cultivation	RT-PCR
		Influenza A + Influenza B
All samples in both seasons	217	217
Positive samples	4	14 + 6

diagnosis. Differential diagnosis was done in selected samples.

Our findings show that all samples positive for cultivation and majority of PCR positive samples were detected during the 2005/2006 influenza season in which the epidemic lasted from the 7th till the 15th week with the peak in the 13th week, except the two samples diagnosed in December and in January respectively.

During 2006/2007 influenza season the epidemic lasted from the 2nd till the 8th week with the peak in the 5th–6th week. All cultivation positive samples and majority of PCR positive samples were detected during this period, except of two samples diagnosed in January and in March respectively.

Comparing cultivation and RT-PCR methods for detection of influenza viruses in biological material samples show us some differences (Table 5). Reasons for these differences might be bad and delay in sample collection, or the delay in delivery of samples to the laboratory or low titer of influenza virus and low virulence in the beginning of the influenza season. In these cases the possibility of detecting the presence of virus by cultivation rapidly decreased. By RT-PCR even small quantities can be detected, so extremely low titers do not pose a problem, but degradation of RNA by enzymes such as RNAse, in the body fluids do pose a problem which can be lowered by strict storing conditions.

High positivity was recorded in samples which were tested for the presence of *Streptococcus pneumoniae* when trying for differential diagnosis of influenza viruses. These results relate with the study of *Streptococcus pneumoniae* prevalence in children attending schools, child care groups and nurseries. A study was carried out to evaluate availability and effectiveness of vaccination against *Streptococcus pneumoniae*.

In conclusion both, conventional RT-PCR and real-time PCR are the useful tools for speeding up the diagnosis and differential diagnosis of influenza viruses. Both methods are very rapid, sensitive and specific. The total time required for processing of samples and evaluating of results reduced from 2–10 days for cultivation to a few hours by PCR. These techniques are efficient tools for effective diagnosis of influenza virus, efficient tools for preventing epidemics and misuse of antibiotics.

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