POSSIBILITIES FOR LABORATORY DIAGNOSIS OF RESPIRATORY SYNCYTIAL VIRUS

T. Hadzhioiova, S. Pavlova, R. Kotseva
Laboratory of Influenza and Acute Respiratory Diseases, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria

ABSTRACT
Respiratory syncytial virus (RSV) is well recognized as the single most important pathogen accounting for acute viral infections of the lower respiratory tract in infants and young children. That is why rapid detection of RSV is mandatory for early diagnosis, isolation measures, and antiviral therapy. The aim of our study was to implement several tests for diagnosis of RSV and determine the role of this pathogen in high risk infants in Bulgaria. During the period 2003-2005 the Laboratory of Influenza and Acute Respiratory Diseases responded the new requirements for the diagnosis of RSV using a complex of classic and contemporary methods. HEp-2 cell lines were used for the isolation of the viruses – total of 7 RSV strains were isolated and identified by classical CFT. Rapid enzyme immunoassays – Directigen were used for the detection of RSV viral antigens of 16 passaged clinical samples. 12 positive by IFA modification on chamber slides were identified. In 2005 RT-PCR using N specific primers was applied for detection of RSV genome in initial samples from patients before viral isolation. The obtained 4 positive results by this method helped to decode the aetiology of the outbreak in Pazardjic in January 2005. Our work shows that the laboratory Influenza and Acute Respiratory Diseases have the readiness for effective diagnosis of RSV using a complex of contemporary methods for detection of RSV in clinical samples.

Introduction
Respiratory syncytial virus (RSV) is the major cause of acute lower respiratory tract infection in children and vulnerable adults (3). RSV is distributed worldwide and, in temperate climates, annual epidemics occur during the winter months. Severe RSV bronchiolitis and pneumonia requiring hospitalization typically occur in infants less than 9 months of age (9). RSV is the most common cause of bronchiolitis. Children with underlying illnesses such as congenital heart disease and bronchopulmonary dysplasia are at increased risk for severe infections due to RSV. In addition, RSV is increasingly recognized as an important pathogen in other groups, including immunocompromised patients and the elderly (2,4).

RSV is a member of the genus Pneumovirus within the family Paramyxoviridae. It has a nonsegmented, negative-stranded RNA genome (3). Thus, it does not have the capacity for reassortment of genome segments, the process by which influenza virus undergoes antigenic shifts leading to influenza virus pandemics. The RSV genome encodes the synthesis of at least 10 viral proteins. There are three transmembrane glycoproteins, i.e., the attachment glycoprotein (G), the fusion protein (F), and the small hydrophobic protein (SH). There are two matrix proteins, M and M2 (or 22K); three proteins associated with the nucleocapsid- N, P, and L; and two nonstructural proteins, NS1 and NS2.

Two viral proteins, the attachment glycoprotein G and the surface glycoprotein F,
are the main antigens responsible for inducing a neutralizing immune response and resistance to infection (3). Two antigenic groups of RSV (groups A and B) have been described based on their reactivity with monoclonal antibodies by Anderson et al., (1985); Mufson et al., (1985); Hall et al., (1990).

Rapid detection of RSV is mandatory for early diagnosis, isolation measures, and antiviral therapy. Several rapid diagnostic methods, including conventional cell cultures, enzyme immunoassay (EIA) and immunofluorescence assay (IFA), based on the detection of RSV antigen in respiratory secretions, have been increasingly used for these purposes. Reverse transcription-polymerase chain reaction (RT-PCR) is a highly sensitive method that has been used successfully for detection of RSV in respiratory samples in children with RSV (5, 10, 11).

The aims of our study were:
• To implement several contemporary tests for the detection of two standard strains of RSV at the earlier stage
• To use these tests for the detection of virus in clinical samples at the later stage.

Materials and Methods

Clinical samples:
Nasal and throat swabs were taken from patients (mainly infants and children up to 2 years of age) with respiratory diseases in acute phase of the disease (till the 3-rd day of the onset).

Standard strains:
Two standard RSV strains were used – one from Lyon, France received in 2002 during the Second European Quality Control (SEQC) and Rendal strain maintained in the laboratory from years.

Cell cultures for viral isolation:
Hep-2 cell lines were used for this assay. The cell suspension was seeded in 24 wells slides (volume 1ml/well). The slides were incubated at 37 °C in CO₂ - atmosphere. The cell monolayers were infected with standard strains and patients’ samples 0,2 ml/well – 2 wells for each sample. One ml/well Minimum Essential Medium (MEM) with 2 % foetal calf serum (FCS) were supplemented to each well. The cell lines were observed for presence of cytopathic effect (CPE) - syncytia formation during 5-7 days.

Rapid Directigen assay:
Directigen RSV are rapid in vitro enzyme immunoassay membrane tests for direct and qualitative antigen detection of RSV in clinical samples (nasopharyngeal swabs). Specific monoclonal antibody enzyme conjugates for RSV antigen were used in the test. Development of a purple triangle on the membrane of the device indicates a positive result for RSV. Total test time is less than 15 minutes.

Modification of the IFA on cell cultures:
Hep-2 cell suspension was seeded in 16 wells chamber-slides (volume 0,25ml/well). The slides were incubated at 37 °C in CO₂ - atmosphere. The cell monolayers were infected with 0,1ml /well standard strains or patients’ samples. 0.2 ml /well MEM was supplemented to each well. The incubation was held for 48h. Dako Cytomation IMAGEN™ RSV kit which contains labelled with FITC conjugated monoclonal antibodies to N and F proteins of RSV was used as previously described (8). The slides were analyzed by fluorescent microscope (magnification - 16 x 0.4). The positive result was regarded as specific when staining in apple-green (in color) within the cells (3 cells minimum/field) against the background of dark red color of the uninocfected cells. The results were compared with positive and negative control samples.

RT-PCR for detection of RSV:
RNA was extracted from the cell culture supernatants containing standard RSV strains and from the clinical samples. The RNA isolation steps followed the same protocol using Trizol LS reagent /Invitrogen, USA/ as described previously for influenza viruses (7). Viral RNA was
Detection of RSV in clinical samples by different methods

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<th>Year</th>
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<td>Directigen RSV</td>
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<td>2003</td>
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eluted in 25µl dd H2O. RT-PCR was carried out using one-step SuperScript RT-PCR (Invitrogen, USA) kit performed at “OPTICON I” (MJ Research, USA) by employing specific primer pair selected from highly conserved regions of RSV N gene. The primers were originally designed to amplify a region between nucleotides 858 and 1135 of the N gene (1):

N1 5’GGAACAAGTTGTTAGGTTTA TGAATATGC3’
N2 5’CTTCTGGCTGCAAGTCTAGTAC ACTGTAGT3’

Distilled water was included as negative control. Twenty ml of the obtained PCR products /amplicons/ were analyzed using 2% agarose gel electrophoresis and their sizes approximately 280 bp were compared with the size of the amplicons received from the standard strain. 100 bp DNA molecular weight marker (Invitrogen) was used in the electrophoresis.

Results and Discussion
1. Isolation of RSV from clinical samples
The use of several contemporary detection methods of RSV began in 2002 with reference to the Second European Quality Control (SEQC) for Influenza and RSV organized by National Influenza Centre – Lyon, France. The identified virus as RSV was used later as a standard strain – called Lyon strain in our current work (6).

During the period 2003 - January 2005 samples from infants were investigated by different methods (Table). Fifty four samples were investigated in total; 31 samples were taken in 2003 from Children Hospital - Sofia; 13 samples were obtained in 2004 from Neonatology Clinic - Sofia; 10 samples were sent to the laboratory in January 2005 from Infant’s house in Pazardjic. Five RSV strains were isolated from HEP-2 cell culture in 2003 and 2 strains in 2005. The strains were identified by classical complement fixation test (CFT) and contemporary methods. The new isolated RS viruses developed characteristic CPE with syncytia formation after 3-7 days incubation period. Most of the viruses were isolated at first passages and gave late CPE (after the third day of incubation). The standard virus strains used as positive controls in the following diagnostic methods showed early CPE (after 48h of incubation) in the same cell cultures. The lack of isolated viruses in 2004-2005 was probably due to the instability and low concentration of the virus in clinical samples.

2. Directigen assays
Rapid in vitro enzyme immunoassay tests are usually recommended for direct detection of RSV in initial patients’ samples. In our laboratory practice we have used modification of Directigen assays only for suspected samples after incubation in cell cultures for 48h.

During 2003-2005 rapid assays allowed early identification of 16 suspected samples. Additional passages of the positive samples result in 7 viral isolates in 2003 and 2005. Directigen was very useful screening test before the introduction of other complementary methods - IFA and RT-PCR for the diagnosis of RSV. (Table, Fig. 1).

3. Modification of IFA on cell cultures
During the season 2003-2004 we applied
for the first time in the laboratory the modification of IFA on MDCK cell cultures for influenza viruses. 743 clinical samples in accordance with Quintiles project were tested (8).

This modification was realized on HEp-2 cell cultures for RSV. In 2003 we performed the test initially with 2 standard strains and in 2004 with clinical samples from 13 newborn and infants. Total of 7 positive results from patients’ samples were received by this technique (Table, Figs. 2, 3). In 2005 five samples were proved to be positive from the total of 10 patients’ samples from the outbreak in Pazardjic and 2 RSV strains were isolated from IFA positive materials.

The modification of IFA allows avoiding the disadvantages from the direct application of the method for clinical samples, where the number of fixed cells depends mainly on the quality of the samples. The specimen inoculation in cell culture helps the replication of the virus for a short period /48h/ increasing the possibility of specific detection even when the concentration in the initial samples is very low. This method is a part of the complex methods used in the laboratory for the diagnosis of RSV. This research is connected with the project “Determination of the RSV role in high risk infants that began in the end of 2004.

4. RT-PCR for RSV detection

RT-PCR was applied for detection of influenza virus type A and B genome in 2002 during the SEQC (6). At this stage primers for RSV were not available in the laboratory. Only in 2004 two samples from the SEQC were confirmed as RSV by RT-PCR. They were used in 2005 as positive controls to be compared with unknown clinical samples with suspected RSV infection. In January 2005 4 positive results for RSV were detected from 10 initial patients’ samples from the outbreak among infants in Pazardjic by RT-PCR.

The RSV aetiology of this outbreak was confirmed by IFA and Directigen in cell culture samples where the virus is already replicated and 2 RS viruses were isolated additionally. For the first time we demonstrated that RSV can be detected in initial samples by RT-PCR.

The visualization of the amplicons of 2 positive RSV clinical samples with sizes corresponding to RSV standard strain.
Fig. 4. Detection of RSV by RT-PCR with N specific primers in initial clinical samples.
Lane №1 - DNA marker /100 bp /
Lane №5 - sample №1 with N primers
Lane №7 - sample №2 with N primers
Lane №8 – negative control - H₂O
Lane №10- positive control – standard RSV strain.

(about 280 bp) are shown at Fig. 4.

Conclusions
During the period 2003-2005 several contemporary tests were implemented for the complex diagnosis of RSV at the laboratory of Influenza and Acute Respiratory Diseases.

Despite that the viral isolation remains the "gold standard" for the diagnosis of any virus and RSV doesn’t make an exception, however in our study the process is not always successful.

The antigen detection tests – Directigen and IFA are considered as rapid and we use them as tentative tests with RSV inoculated cell culture samples.

Due to the possibility of RT-PCR to detect very low RNA copies of the virus we applied this technique for diagnosis of RSV infection in initial patients’ samples.

All these methods used in the routine diagnosis of RSV infection in the laboratory will allow more detailed determination of the role of this virus among high-risk children in Bulgaria.

REFERENCES