RT PCR Detection and Propagation of Respiratory Syncytial Virus in Human Lung Carcinoma Cells (A549) cell line

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Abstract:
In this study negative result of real-time reverse transcription-QPCR (RT-PCR) assay tests of Influenza virus of nasal screetion and throat swap samples of Iraqi patients hospitalized with signs and symptoms of an upper respiratory tract infection in Central Republic Health Laboratory in Iraq were tested for Respiratory Syncytial Virus infection by RT PCR .Positive samples was 4 out 0f 20 were used .Viral isolation was done on a monolayer of 70-80% confluent Human Lung Carcinoma Cells (A549) cell line and incubated at 33ºC for 4 days .Syncytia was observed in 3 positive samples.

Keywords: RT PCR, RSV, Tissue culture.

تشخيص وتنمية فيروس Respiratory Syncytial Virus بتقنية سلسلة تفاعل البلمرة في خطوط خلايا سرطانية لرئة الانسان (A549)

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الخلاصة
في هذه الدراسة تم اخذ 20 عينة من مسحات من افرازات الانف والحلق لمرضى عراقيين راقدين في المستشفيات واللذين يعانون من اصابات في الجهاز التنفسي والتي كانت نتائج فحص سلسلة تفاعل البلمرة الكمي RT- QPCR لفيروس الانفلونزا سالبة .تم فحص هذه العينات للكشف عن وجود اصابة بفيروس Respiratory Syncytial Virus بفحص سلسلة تفاعل البلمرة RT PCR في مختبر الصحة المركزي .كانت نتائج 4 عينات موجبة من مجوع العينات .تم عزل وتنمية الفيروس في خط خلايا رئة سرطانية للإنسان (A549) وحيث تم اصابة طبقة خلايا بكثافة %(70-80) وبعد فترة حضانة 4 ايام في 33 درجة مئوية تمت ملاحظة التأثيرات المرضية المتمثلة بتكون Synsytia في طبقة الخلايا لثلاث عينات موجبه.

Introduction

Every year during the winter season there are many populations is under attack from Human respiratory syncytial virus (HRSV) in UK and United States of America. These viruses infect respiratory tract and cause mild to severe illness. It characteristically changes from year to year, occasionally producing pandemics. The majority of children infected with RSV under 1 year of age develop mild upper

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respiratory tract symptoms. However, up to 40% develop lower respiratory tract symptoms and 0.5–2% of all infants requires admission to hospital. Most RSV-associated bronchiolitis occurs in young children 2–5 months of age. It is unusual in children less than 1 month and in children older than 2 years of age. Of children hospitalized, 1–2% requires intensive care and, in these, mortality can reach 10% [1]. Death from bronchiolitis is rare in children without underlying cardiorespiratory or immunological conditions, and the vast majority of children infected with RSV make a full recovery. However, some infants with RSV bronchiolitis subsequently develop recurrent episodes of wheeze and cough, suggestive of asthma [2]. RSV spreads easily by direct contact, and can remain viable for a half an hour or more on hands or for up to 5 hours on countertops. Childcare facilities allow for rapid child-to-child transmission in a short period of time [3].

In order to track this virus, clinical virology laboratories have traditionally used tissue culture cell lines. The cells used have to be readily infected by these viruses to isolate them from patient specimens. The cell lines amplify the amount of virus present, express the viral antigens and in many cases die as a consequence of the viral infection producing characteristic cytopathic effects in the cell monolayer. The amplified viruses are then available for further identification by molecular techniques to determine whether they are common or new strains of the virus [4].

HRSV is an important viral agent of respiratory tract disease in infants, children, immunosuppressed individuals, and the elderly [5]. In the absence of a vaccine, the prevention and treatment of HRSV disease remain a significant challenge. HRSV is a single-stranded negative-sense RNA virus of the family *Paramyxoviridae*. Its genome expresses 11 known proteins, among which are three transmembrane glycoproteins (the small hydrophobic protein SH, G, and F) and the viral M protein [6]. G is a highly glycosylated protein that is expressed as a secreted form and a membrane eanchored form, with the latter serving as a viral attachment protein .F resembles the prototypic paramyxovirus fusion protein but can induce membrane fusion in the absence of G [7]. F also appears to play a role in viral attachment, and nucleolin was identified as a cellular receptor for the F protein [8]. HRSV infectivity is associated predominantly with a filamentous form. These viral filaments are produced at the cell surface late in the infection cycle and remain largely attached to the infected cells [9]. A variety of perturbants were employed to characterize the cellular processes involved. We found that immediately after binding to cells RSV activated a signaling cascade involving the EGF receptor, Cdc42, PAK1, and downstream effectors. This led to a series of dramatic actin rearrangements; the cells rounded up, plasma membrane blebs were formed, and there was a significant increase in fluid uptake [10].

The goal of this study is to detect infections with RSV in Iraqi patients by using RT PCR technique and isolate the virus from nasal secretion and throat swap specimens from patients hospitalized with signs and symptoms of an upper respiratory tract infection in (A549) to see the cytopathic effect in monolayer and prepare virus stock which can be used in further experiments .

Materials and methods

Samples were collected in 5 ml of transport medium (phosphate-buffered saline containing 10% glycerol, 1 mg/ml gentamicin together with 8 IU/ml of penicillin, 8 µg/ml of streptomycin, and 0.02 IU/ml of amphotericin B [Invitrogen, Carlsbad, CA] and stored at 4°C**.** Specimens were processed and tested with a Directigen Flu A kit (Becton Dickinson,Cockeysville, Md.).Negative result samples were tested for RSV infection by RT PCR assay. Subsequent viral isolation performed with specimens with a positive result samples.For tissue culture A549 cell line was provided from durham university /Biochemitry bepartmant .Extraction of RNA from samples was done by using RNeasy Mini kit from QIAGEN and the procedure was done according to the kit instractions.

RLT 350 µl buffer was added and was mixed by pipet. The lysate was Pipeted directly into a QIAshredder spin column was placed in a 2 ml collection tube, and was centrifuged for 2 min at full speed. Ethanol (70%) 1 volume was added to the lysate, and was mixed well by pipetting. The sample (700 µl) was transferred to an RNeasy spin column placed in a 2 ml collection tube. The lid was closed gently, and was centrifuge for 15 s at 8000 x *g* (10,000 rpm).The flow-through was discarded. RW1 Buffer (700 µl)was added to the RNeasy spin column. The lid was closed gently, and was centrifuged for 15 s at 8000 x *g* (10,000 rpm) to wash the spin column membrane. The flow-through was discarded. Buffer RPE (500 µl) was added RPE to the RNeasy spin column and was centrifuged for 15 s at 8000 x *g*

(10,00 rpm) to wash the spin column membrane. The flow-through was discarded. The RNeasy spin column was placed in a new 1.5 ml collection tube. RNase-free water (30–50) µl was added directly to the spin column membrane. , and was centrifuged for 1 min at 8000 x *g* (10,000 rpm) to elute the RNA.

RT PCR assay

QIAGEN One Step RT-PCR kit was used for detection the infection with RSV following the instructions provided with the kit. The forward of N gene primer was 5′ CATCCAG CAA ATA CACC ATCCA 3′ and the revers primer was 5′ GCATCTCTGAGTATTTTTATGG 3′. Template RNA, primer solutions, dNTP Mix, 5x QIAGEN Onestep RT-PCR Buffer, RNAase-free water and 5x Q-solution and were thawed and placed on ice .The reaction mix was prepared according to table 1 according to kit instructions. The reaction mix contains all the components except the template RNA. a negative control (without template RNA) was prepared.

Table 1-Reaction components for one-step RT-PCR using Q-Solution

Component	Volume/reaction	Final concentration
Reaction Mix QIAGEN OneStep RT-PCR Buffer,5x	10 µl	1×;2.5 mM Mg^{+2}
dNTP Mix (10 mM each)	2 µl	400 µM of each dNTP
Primer A	1 µl	0.6 µM
Primer B	1 µl	0.6 µM
RNase -free water	19 µl	_
QIAGEN OneStep RT-PC Enzyme Mix	2 µl	_
5x Q-Solution	10 µl	_
Template RNA	5 µl	_

Template RNA was added (2 µg/reaction) to the individual PCR tubes. PCR tubes are still on ice. Wait until the thermal cycler had reached 50°C. Then place the PCR tubes in the thermal cycler. The thermal cycler was programmed according to the program outlined in table 2. The loading (bromophenol blue) dye was added to each tube. The samples were loaded on to agarose gel and the gel subjected to electric field 5 to 8 V/cm. The gel visualized by staining with ethidium bromide, which intercalates into the major grooves of the DNA and fluoresces under UV light.

Table 2-Thermal cycler conditions

Step	**Time**	**Temperature**
Reverse transcription	30 min	50°C
Initial PCR activation	15 min	95°C
3-step cycling		
Denaturation	0.5–1 min	94°C
Annealing	0.5–1 min	50°C
Extension	1 min	72°C
Number of cycles	35	
Final extension	10 min	72°C

Isolation of RSV in A549 cell line:

Cells and virus manipulation:

All cell and virus manipulation was undertaken in a class II laminar flow hood using latex or polyvinyl gloves. A confluent 175 cm^2 monolayer of A549 was Trypsinised and spilt 1:4 into new 175 cm^2 tissue culture flasks to attain 80-90% confluence within 24 hours. Virus from positive samples was Add the (50 ml and the flasks was shacked gently to ensure that the inoculums reach all parts of the monolayer. Incubate the flasks at 37°C in the humidified CO_2 incubator for 2 hours, with intermittent shaking every 15 minutes. The growth medium was removed from flasks by inversion into a sterile beaker. Maintenance medium was added (10) ml and was the flask incubated at 37°C in the humidified CO_2 incubator for 4-7 days. Small syncytia was evidenced after 4 days infection. Large syncytia were evidenced and the monolayer was detached ,the monolayer was scraped with a sterile rubber policeman . The flask's contents were transferred into a sterile 250 ml conical centrifuge tubes. The suspension was sonicated for 5 minutes at room temperature. Centrifugation was done for 10 minutes at 1000 rpm at 4°C. The supernatants was collected as the virus stocks and were into the 2 ml cryotubes and were stored at (-80).

Results and discussion

RT PCR Detection of infection with RSV gave positive results to 4 patient samples out of 22 samples with RT PCR negative results of influenza virus infection. we used RT-PCR because it is a reasonably sensitive and highly specific method for the diagnosis of RSV infection [11].

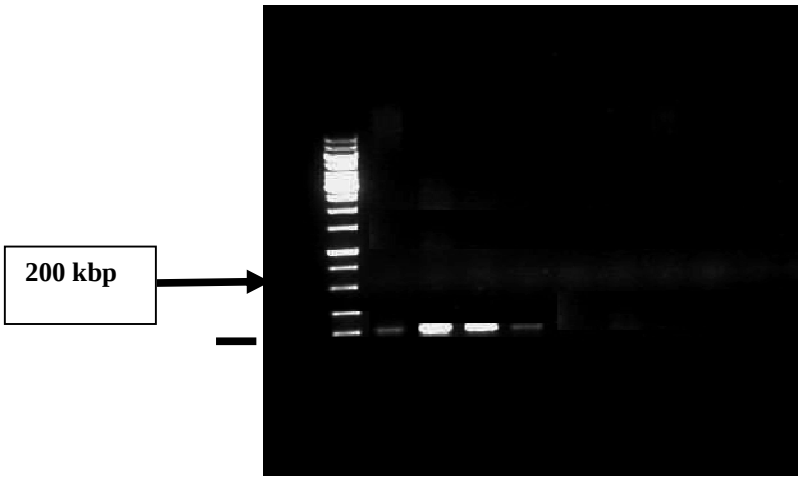

Figure 1- agarose gel analysis of RT PCR of RSV (N) gene.
agarose gel 0.8 gm/ml at 5 V/cm for 1 hour in TAE buffer. The gel was exposed to UV using UV transilliuminator .

Isolation of RSV was successfully done in A549 cell line with 3 samples Positive results of RT PCR for RSV as there were characteristic cytopathic effects observed in Monolayer of A549 cells after incubation more than 4-5 days at 37°C in the humidified CO_2 incubator as shown in figure 3 in contrast with figure 2 which represent normal cells (without infection). In a study different response patterns were observed, with RSV infection of primary AEC cultures causing distinct peaks of viral replication and matched cytotoxic responses [12].

This finding shows that the rate of identification of viral infections in humans by viral culture is lower than that detected by PCR and RT-PCR .this result is agree with a study of Nijhuis M. *et.al* in 2005 which showed the higher sensitivity of the PCR technique than viral culture , but on other hand viral culture may also reflect cases when virus establishes an abortive infection in which viral genetic material is replicated, but production of infectious viral particles is impaired. Undetected by viral culture, this type of replication can only be documented by measuring viral gene expression and genome replication in infected individuals and laboratory animals by PCR [13].

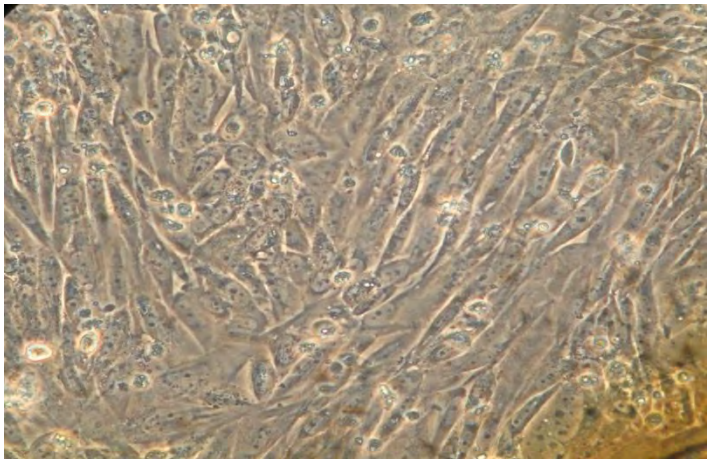

Figure 2- Non infected A549 cells under inverted microscope (400X).

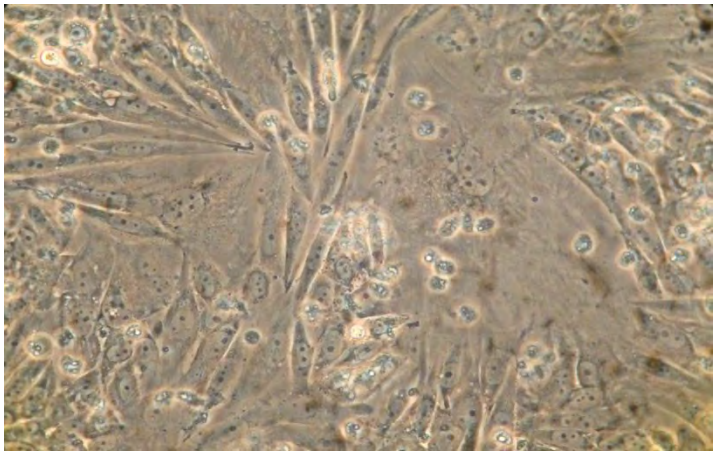

Figure 3- A549 cells infected with RSV under Inverted microscope (400X).

Viral culture and PCR may reveal important features of viral biology, such as an abortive replication as in the case of respiratory syncytial virus (RSV) infection. Viral culture has long been considered the "gold standard" for laboratory diagnosis of respiratory viral infections in humans and for analysis of viral replication in animal models. Over the years, other techniques such as PCR have proven to be specific and sensitive methods of viral detection, with reverse-transcription PCR (RT-PCR) particularly well-suited for analysis of respiratory RNA viruses [14,15].

The use of RT-PCR for investigation the infection with HRSV provide the means to perform accurate epidemiological studies as well as to investigate the immune response to infection .Detection of the infection by viral culture is lower in sensitivity but it can be useful for study the life cycle and further experiment such as testing of novel drugs .

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