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ORIGINAL ARTICLE

Isolation and Identification of Human Rhinovirus from aborted Women in Iraq

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ABSTRACT

Objective: The relationship between pregnant women with Bad Obstetric History (BOH) like abortion and dead fetuses and the infection with Human Rhinovirus (HRV) for the first time.

Methods: Three hundred throat swabs were collected from pregnant women with (BOH) from Mosul and Baghdad , extraced viral RNA from Throat swabs specimens using QIAGEN QIAamp®Viral RNA Min Kit.Germany .while Real-time PCR process for HRV was using (Fast-track FTD Respiratory pathogens 33. Luxembourg). HeLa cell line used for isolation the HRV and after the obtained the CPE and detection the HSV using TEM.

Results: Our results showed that 10% of the collected specimen were positive for the detection of (HRV) by Real Time PCR .The HRV were isolated in continuous human cell lines HeLa Cells Which detected by the appearance of characteristic cytopathic effect (CPE), which initially appears as foci of rounded up cells. The diagnosis of HRV was confirmed by using Transmission Electron Microscopy (TEM), which showed positive appearance of HRV as small round in shape its size about of 30.5 nm at 200 kx.

Conclusion: There are a correlation between HRV infection with pregnant women with BOH and which diagnosed it by Real time PCR and tissue culture and determined it by TEM.

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INTRODUCTION

Human Rhinovirus (HRV) are small, nonenveloped RNA viruses classified as picornaviruses[1]. There are three large groups of human pathogens among the Picornaviridae family: the Enteroviruses (Enteroviruses, Polioviruses, Coxsackie viruses, and Echoviruses), the Hepatoviruses (Hepatovirus A), and the Rhinoviruses[2]. Respiratory virus infections are a major issue during pregnancy, since pregnant women have increased susceptibility to respiratory virus infections as well as subsequent increased disease severity and duration of infection. Pregnancy can be viewed as an immunological balancing act where fetal tolerance (via maternal immunosuppression) is essential and yet a degree of maternal protection must be maintained

against invading pathogens[3]. Rhinovirus was the most commonly detected pathogen among pregnant women in the developing countries. This finding is notable because Middle East respiratory syndrome Coronavirus (SARS) and severe acute respiratory syndrome coronavirus during pregnancy have been found to be associated with severe complications and illness in the woman and the infant[4, 5, 6].

Direct detection of HRV by culture or PCR . Antibodies against viruses can be measured by serological methods such as enzyme-linked immunosorbent assay (ELISA).Serological assays are diagnostically useful only when the HRV serotype is already known[7, 8].

Primary cells such as human embryonic kidney, bronchial epithelial, tonsil, and continuous human cell lines such as HeLa, H292, and HEP-2 can support growth of HRVs. The most commonly used cells for cultivation Rhinovirus are the WI-38 strain and the MRC-5 strain of diploid fibroblasts, foetal tonsil cells, and HRV-sensitive HeLa e.g., Ohio HeLa[9, 10].

Nevertheless virus culture in susceptible cell lines has been the 'gold standard' for laboratory diagnosis of respiratory virus infections. However tissue culture techniques are generally laborious, time consuming, and insensitive when compared with more recent PCR-based assays[11].

MATERIALS AND METHODS

Specimen collection

Three hundred throat swabs from women with BOH like abortion and dead fetuses from Mosul and Baghdad hospitals, inclusion Mosul City: AL-Salam Teaching Hospital, Al Khansa Teaching Hospital for Maternity & Children and Al- Batool Hospital for Gynaecology & Obstetrics and were in Baghdad City: Al Alwaiya Maternity Teaching Hospital, Al-Kademia Hospital for Children , AlYarmuk Teaching Hospital and during two years. The Throat swabs of samples were placed in sterile Viral Transport Media (VTM) and directly transported laboratory and stored at -70°C Deep freeze until used in viral RNA extraction.

Viral RNA extraction

The extraction of viral RNA from Throat swabs specimens using QIAGEN QIAamp®Viral RNA Min Kit.Germany according to manufactures[12]. This kit provide the fastest and easiest way to purify viral RNA for reliable use in amplification technologies. Viral RNA can be purified from plasma (treated with anticoagulants other than heparin), serum, and other cell-free body fluids.Samples may be fresh or frozen, but if frozen, should not be thawed more than once.Repeated freeze–thawing of plasma samples will lead to reduced viral titers and should be avoided for optimal sensitivity. Cryoprecipitates accumulate when samples are subjected to repeat freeze–thaw cycles. This may lead to clogging of the QIAamp membrane when using the vacuum protocol. QIAamp Viral RNA Mini Kits are for general use and can be used for isolation of viral RNA from a wide variety of viruses, but performance cannot be guaranteed for every virus.

Real-time PCR process

Polymerase chain reaction (PCR) techniques have advanced further with the development of quantitative real-time PCR (qRT-PCR). This technique is more sensitive than conventional PCR and enables the number of viral RNA copies in a sample to be measured. For this RNA was extracted from potentially infected samples and copied to cDNA by reverse transcription. PCR using HRV-specific primers targeting conserved viral sequences, such as the 5′-UTR, is then performed to determine the presence of viral genetic material in the original sample , the viral RNA is transcribed into cDNA using a specific primer mediated by reverse transcription step followed immediately in the same tube by polymerase chain reaction using (Fast-track FTD Respiratory pathogens 33. Luxembourg). The majority of picornaviruses detected were Rhinoviruses; the FTD assay was the only assay to differentiate rhinovirus within the picornavirus family[11].

The presence of specific pathogen sequences in the reaction is detected by an increase in fluorescence observed from the relevant dual-labeled probe, and is reported as a cycle threshold value (Ct) by the Real-Time thermocycler. The assay used Equine arteritis virus (EAV) as an internal control (IC), which is introduced into each sample and the negative control at the lysis buffer stage of the extraction process by using Applied Biosystem Real time PCR 7500. Germany[13].

Fast-track mastermix PCR programme:The first holding stage at 50°C for 15 minutes hold , Second holding stage at 94°C for 1 minute hold , and the cycling stage 40 cycles of 94°C for 8 second and in cycle 2 at 60°C for 1 minute with data collection.

Tissue culture propagation of human Rhinovirus

Throat swabs of VTM were filtered by syringe filters, 0.22µm, to remove any other contaminated microorganisms like bacteria and Mycoplasma . HeLa cell line from (Al-Mustansiriya University / Iraq Center for Genetics and Cancer Research) used for virus cultivation.Basic media was prepared by add 5.5 ml from Penicillin – Streptomycin 100x and 5.5 ml of L-glutamine 200 mM to 500 ml of DMEM (Dulbecco's Modified Eagle's Medium) High glucose (4.5 g/l), with 25 mM HEPES, without L-glutamine. The growth media was obtained by adding 10% of Fetal Bovine Serum Standard Quality to basic media while 1% of the same serum was added to obtain maintenance media.

Inoculation of cell cultures

Complete monolayer Hela cells propagated in 25 ml flasks were used for inoculation 1ml of processed specimen of suspected HVR .

All inoculated flasks were incubated for one hour at 37 C° for virus adsorption, then 7 ml of DMEM maintenance media was added to each flasks as well as two non inoculated complete monolayer flasks remain as negative control. Then all flasks were observed daily for (7-10) days for the detection cytopathic effects[14, 15].

Transmission Electron Microscopy (TEM) examination

All positive HRV samples resulted by Real time PCR were inoculated in tissue culture (Hela cells) the inoculated cells which show clear CPE were used for TEM in AL-Nahrain University/ College of Medicine/ Department of Human Anatomy.

The virus suspension was clarified using cool microfuge at 2000xg for 10 min twice. The supernate was discarded and the remaining pellet was added to it formaldehyde or glutaraldehyde (20 min, final concentration 2% and 0.5%, respectively) as a fixative[16]. Formvar solution 5% (5 gm free flowing powder/95 D.W) was immersed in distilled water and forming a floating membrane on the surface of water.

Transmission Electron Microscope (TEM) copper 200-mesh grids were placed by a watchmaker forceps on the floating membrane of formvar solution 5%, and retrieved by pushing down on the grid by a filter paper.

For negative staining, a drop of about 10 µl of the virus suspension is applied to the hydrophobic surface of a parafilm square in a Petri dish.

A formvar-coated grid was floated onto the virus suspension drop for one minute to hold the small particles, with the formvar side of the grid in contact with the liquid. The grid was immediately floated in a drop of 1.5% phosphotungsic acid , and1% aqueous uranyl acetate. For a better assessment of the samples, three grids were prepared for each sample. After staining for one minute, the excess stain was removed with filter paper and the grid was left to dry for a few minutes. Insertion the grid into the microscope column TEM[17].

RESULTS

The mean age of Rhinovirus infection in pregnancy women with BOH is 27±6.8. Thirty throat swaps (10%) out of 300 samples were indicated positive HRV using Real Time PCR (Table 1) which amplification reaction appeared as specific detection of HRV at High Ct value 27.8 while the The mean Ct value of Rhinovirus infection 30±1.6 (Figure 1), from these positive thirty samples HRV was firstly isolated from pregnant women in Iraq by tissue culture propagation , the isolated virus show clear CPE three days post inoculation. The CPE of isolated HRV on Hela Cells characterized by rounding and more refractile (brighter) as well as different changes in size of infected cells as noticed under inverted microscope.

Transmission electron microscope results show appearance of HRV small round shaped its size about of 30.5 nm at 200 kx (Figure 2).

Table 1: **The relationship between blood group , Age of Human Rhinovirus infection.**

Age/Year		Blood Groups				
		A^+	B^+	AB^+	O^+	O^-
Under 20	4	-	-	-	3	1
20 - 29	17	2	2	4	9	-
30 - 39	7	1	1	3	2	-
Above 39	2	-	-	-	1	1
Total	30	3	3	7	15	2
	%	10	10	23.3	50	6.7

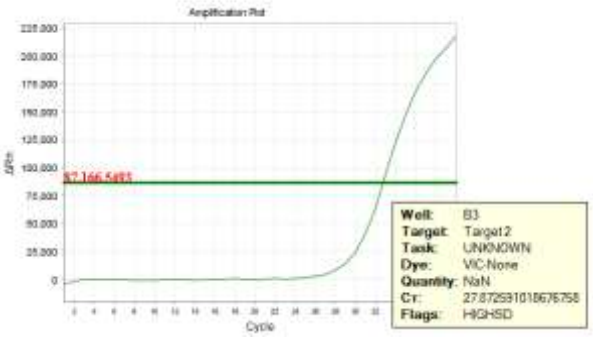

Figure 1: **Amplification plot showed the amount of fluorescence obtained in each amplification cycle. A Ct was 27.8 that account of HRV.**

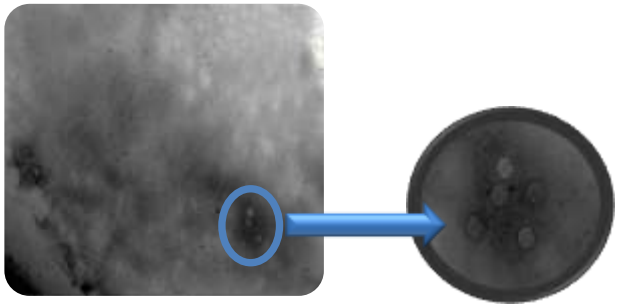

Figure 2: **View of TEM field stained with phosphotungsic acid and aqueous uranyl acetate negative stain to show the HRV particles.**

Discussion

The most useful methods for detection HRV in clinical samples was Real time PCR[13, 18].

Tyrrell and other 1968 showed that the relationship between blood group and common cold infection like Rhinovirus that persons of blood group O more than infected to those of other groups, that conformed with our work[19, 20].

Results of our study in Real Time-PCR were positive (below Ct 33) Van Regenmortel and Mahy, (2010) indicate that the HSV Ct is (27,26) which confirmed our study and explain that Real-time PCR based on the use of melting temperatures, allows simultaneous detection and analysis of several different nucleic acids. It also allows testing for more than one virus from the same sample[21].

Other Researchers and studies were indicated the relationship between HSV and BOH the studyof Pilorgé and other ,(2013) that Rhinoviruses were also the first non-influenza viruses responsible for influenza-like illness in a series of 78 pregnant women sampled in 2009–2010, While Philpott and other, (2017) showed the HRV during pregnancy was associated with an increased risk of low birth weight (LBW), Because low birth weight infants have an increased risk of mortality compared with their heavier counterparts and HRV is a highly prevalent respiratory virus, this may represent a potential modifiable risk factor to reduce risk of LBW infants, particularly in developing countries. Interventions to reduce the burden of febrile

respiratory illness due to Rhinovirus during pregnancy, such as measures to improve infection control, development of targeted therapies, or improvement of diagnostics, may have a significant impact on LBW and subsequent infant mortality worldwide[11, 22].

The cytopathic effect (CPE) of human Rhinovirus infection on HeLa cells when we isolated virus on the tissue culture , that Uninfected cells are adherent, they normally grow flat and stuck down firmly on the tissue culture flask. After infection with Rhinovirus, the cells change shape, becoming round and more refractive (brighter) under inverted microscope, Some infected cells detach from the tissue culture flask and float in the medium[23].

Hao and other (2012) confirmed that the cultured of Human Rhinovirus on HeLa cells and Many viruses can be identified by the characteristic cellular changes they induce in susceptible cell cultures. These can be visualized under the inverted microscope. The degree of CPE is usually graded from + to ++++ based on the percentage of the cell monolayer infected: 25% of the cell monolayer (+) one few , 50% (++), 75% (+++), and 100% (++++) that's mean all except few cell attached . There are two important points that should be emphasized regarding CPE induced by virus (Figure 3):

1. The rate at which CPE progresses may help to distinguish similar viruses.
2. The type of cell culture (s) in which the virus replicates is important factor in identification[24, 25].

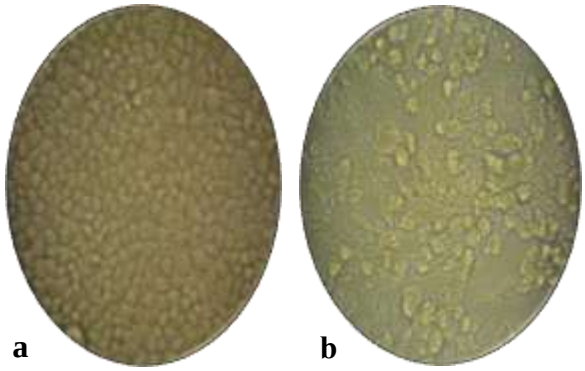

Figure 3: **Hela Cells under Inverted Microscope a: Control without inoculation. b: Inoculated with HRV and show the CPE.**

In developed countries, electron microscope EM has been been considered an efficient tool for direct detection of viruses through visualization of the viral particles in body fluids . The identification is based on morphological characteristics specific to each virus family and requires a certain amount of viral particles up to 10^6 particles/ml[26].

Our results show the HRV of 30.5nm in size which in agreement with Pickl-Herk and other in 2013[27]. The combination of EM with culture-based methods has shown great contribution in the diagnosis of viral infections, along with serology testing for detection of antibodies targeted against the virus. These conventional methods are still fundamental practices in many hospital laboratories, TEM is an analytical tool allowing visualisation and analysis of specimens in the realms of microspace 1 micron/1µm = 10^{6}m to nanospace 1 nanometer/nm = 10^{9}m[28, 29].

This technique was more confirmation about the Human Rhinovirus as the diagnosis process conformable with Hewat & Blaas 1996 and Olson and other 1993 which show and confirmed HRV under electron microscope and determined the particles of the virus[30, 31].

Conclusions

Our study showed the relationship between Human Rhinovirus and abortion and dead fetuses in pregnant women with BOH, and the Importance of investigated the molecular epidemiological characteristics of HRV in pregnant women by Real time PCR and confirmed that by Cultivation in continuous Hela cells and identification by TEM.

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