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RESEARCHARTICLE

DETECTION FOR RESPIRATORY SYNCYTIAL VIRUS RNA AMONG CHILDREN WITH FLU-LIKE ILLNESS USING MOLECULAR ASSAY

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ABSTRACT

Respiratory syncytial virus (RSV) is one of the most common causes of lower respiratory tract infection in the developing world. Viral culture had low sensitivity for viral detection from PCR method lead to reduction in viral identification. In Iraq, little data are available for RSV detection, previous studies restricted on cell line culture and ELISA techniques. Hence, the current study aimed to investigate RSV-RNA in respiratory secretion with RT-PCR assay. One hundred children under 5 years with flu-like illness and negatively for influenza virus A and B types were tested for RSV.The real-time and conventional RT-PCR detection of RSV RNA were reported in 19% and 6%, respectively.The significant observed of RSV infection in children less than two years old. Moreover,the severity of disease was a significant increase in viral quantity from other RSV infection with mild disease.Sensitivity of conventional RT-PCR has not detected the positive cases in 68.4% of RSV infections at real-time RT-PCR assay in which the increasing of viral load were significant compared with those positive detectable in conventional RT-PCR. This study concludes a using multiplex PCR is important for diagnosis of RSV along with influenza virus, addition to human rhinovirus and the human met pneumonia virus as a potential infection of influenza like-illness which occur in the second most common cold pathogen.

INTRODUCTION

Respiratory syncytial virus (RSV) is one of the most common causes of lower respiratory tract infection in the developing world (Boyce *et al*., 2000). The RSV strains are separated into two major groups (A and B) on the basis of antigenic and genetic variability. The RSV A was the predominant group in both nosocomial and community-acquired RSV patients (de-Paris *et al*., 2014). Most significantly, RSV infection was associated with a 3-fold increase in bacterial detection like Streptococcus pneumonia (Chapell *et al*., 2013). The RSV load was the strongest independent predictor of disease severity (DeVincenzo *et al*., 2005). Symptoms begin near the time of initial detection, peaked in severity near when viral load peaked, and subsided as viral load slowly declined. Increased viral load correlated consistently with increases in multiple different disease measurements (DeVincenzo *et al*., 2010). Other investigators documented that independently associated of the following risk factor with viral load at that time: household crowding, age at admission, sex ethnicity and use of day care (El Sabeeby *et al*., 2011). Real-time RT-PCR has several advantages from traditional methods of viral detection and to other PCR technologies (Rohde *et al*., 2003), one of them is measured by the viral load. Low detection rates in culture as compared to real-time RT-PCR could be explained based on the explanation described previously on difficulty of RSV due to itsthermolability (Falsey *et al*., 2002). It is likely the PCR results indicate to the presence of viral RNA or noninfectious viral particles (Van de Pol *et al*., 2010).Although the viral culture has been thought to be the gold standard for their testing, it is generally slow and often taking up to 14 days before the results are available (Rudan *et al*., 2008). Furthermore, this assay had low sensitivity for viral detection from PCR method due to reduction in viral identification. In Iraq, little data are available for RSV detection, previous study restricted on cell line culture and ELISA techniques. Hence, the current study aimed to investigate RSV-RNA in respiratory secretion with RT-PCR assay.

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MATERIALS AND METHODS

Specimens

A total of 100 swabs for each nasopharyngeal and throat from 100 children aged from 3 months to five years with flu-like illness were collected in 2 ml of transport media and stored at -20C° until use. All samples were obtained from out patients during the period from Oct. to Dec. 2013, since the first week of clinical signs from mild including rash, cough, and fever to sever such high fever, admission to the intensive care unit, the need for mechanical ventilation and bronchitis (ElSaleeby *et al*., 2011). Previously, these samples checked negatively for

influenza virus type A and B by applying real-time RT-PCR with specific primers and probes.

RNA Extraction

RNA was extracted from both nasopharyngeal and throat swabs by mixing 500µl of them and 300µl was taken from mixing to RNA extraction using QIA amp Viral RNA Mini Kit (QIAGEN), according to the manufacturer's instructions. The RNA extracted was stored at -70C° until use for both real-time and conventional RT-PCR.

Real-Time RT-PCR

For RSV F gene detection, used primers and probe were described by another study for both RSV A and B detection (Mentel *et al*., 2003). A forward primer sequence 5′-AACAGATGTAAGCAGCTCCGTTATC-′3, reverse primer 5′-CGATTTTTATTGGATGCTGTACATTT-′3 and probe 5′-TGCCATAGCATGACACAATGGCTCCT-′3 that labelled with 5′ reporter dye FAM and the′3 quencher dye TAMRA. In one step RT-PCR, master mix reagents (QIAGEN) were added to 10 µl RNA templates, 0.5 pmol conc. of each primers and 0.3 pmol conc. of probe in 20 µl reaction mixture. Amplification and detection were done with an Applied Biosystem7500. Briefly, one cycle for 30 min at 50C° and 15 min at 94C°, followed by 45 cycles for 10 s at 95C° and 1 min at 60C°.

Conventional RT-PCR

One step RT-PCR was assayed for RSV using the same describedin real-time RT-PCR. After optimization of the primer concentration, a positive samples by real-time RT-PCR were carried out in a 50 µl reaction mixture containing 10µl of template RNA, 2 µl of enzyme mix and 10 µl of buffer 5x (QIAGEN one step RT-PCR), 0.6 µM conc. of each forward and reverse primers for RSV, and 400 µM conc. of each dNTP. ThePCR amplification protocol was performed as follows: 30 min at 50 C° for reverse transcription reaction, followed by 10 min at 95C° for initial Taq NA polymerase activation, and 40 cycles of 30 s at 95C°, 1 min at 55C° and 1 min at 70 C° then final extension 5 minat 70 C°. The amplicon electrophoresis with ethidium bromide staining on 2% agarose gel was done.
Statistical Analysis

All data were tabulated and analyzed using the SPSS IBM version 20. The Chi-Square test was done to investigate probable correlation between patient information and RSV infection, while the Mann-Whitney (U-test) used to find the significant role of Ct values in disease severity and sensitivity of conventional RT-PCR for infection diagnosis. Values were considered statistically significant $P \leq 0.05$.

RESULTS

In the current study, the real-time and conventional RT-PCR detection of RSV RNA were reported in 19% and 6%, respectively of children under five years old with flu-like illness, and who negatively to influenza virus type A and B diagnosis. Moreover, the significant observed of RSV infection in children less than two years old when compared to other studied age groups as shown in Fig. 1 (X^2 =6.42, P< 0.05). Approximately equally of RSV infections in both male and female have been shown. By results of real-time RT-PCR, this finding gave away that endpoint of positive samples with a threshold cycle (Ct) value should be less than 39 (Fig. 3).

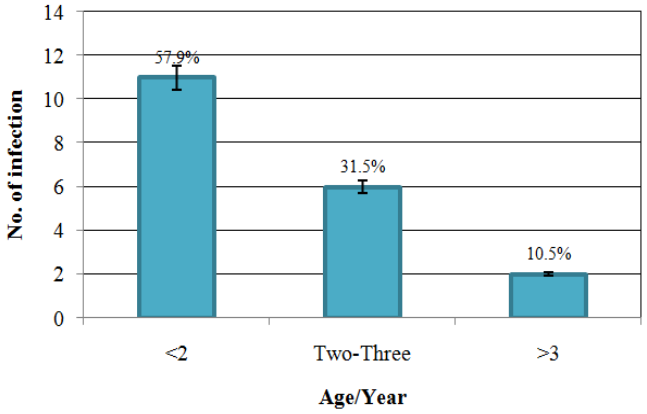

Fig. 1. RSV infections distribution among children with flu-like illness who's negative for influenza virus type A and B, more than half infections in children fewer than two years were appeared. Asterisk indicates to significant increase compared to other ages (P<0.05)

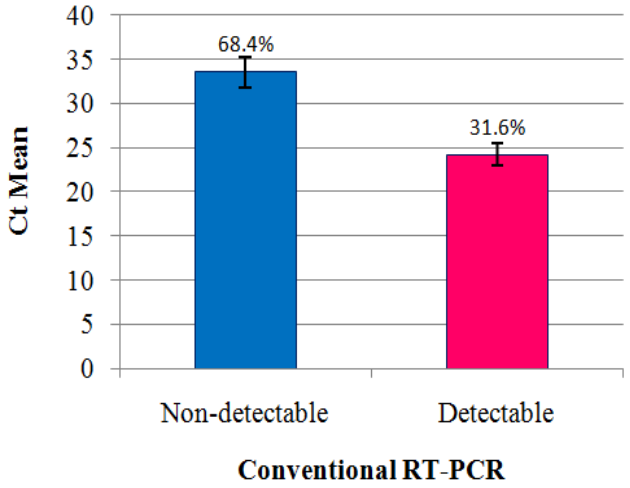

Fig. 2.Mean of Ct values in 19 positive samples for RSV where identified with real-time RT-PCR among children have flu-like illness. Of these, 68% was not detect in conventional RT-PCR in which the increasing of Ct values were significant compared with those positive detectable in conventional RT-PCR (P<0.05)

Regarding to the viral quantity of clinical sample, finding give a hint that the Ct values which indicate to the viral load in the detected sample. However, the Ct values among studies ages were not statistical significant (P>0.05), although most infected children less than two years old had lowered Ct values (high load) from other ages, occurrence of Ct values between 20.5 and 37 have been recorded. Furthermore, Ct values in both sexes of infected children have not occurred significantly (U-test= 39, P=0.62). Meanwhile, the severity of disease was a significant increase in Ct values from other RSV infection with mild disease (U-test= 10.5, P<0.01).Sensitivity of conventional RT-PCR has not detected the positive cases in 68.4% (Fig. 2) of RSV infections at real-time RT-PCR assay in which the

increasing of Ct values were significant compared with those positive detectable in conventional RT-PCR (U-test=8, P<0.05).

Figure 3 demonstrates some positive cases with low Ct values less than 30 in real-time RT-PCR and their amplicon in conventional RT-PCR have been shown a specific band with 90 bp in agarose gel (Fig. 4).

sample with Ct value over 29 was not detected in conventional RT-PCR. A higher it's from conventional RT-PCR sensitivity was observed to detect 10 copies/ml and 8×10^3 copies/ml, respectively in the sample (Bharaj *et al*., 2012). Furthermore, it has been advocated over conventional methods due to its advantages including sensitivity, specificity and speed (Weinberg *et al*., 2004).

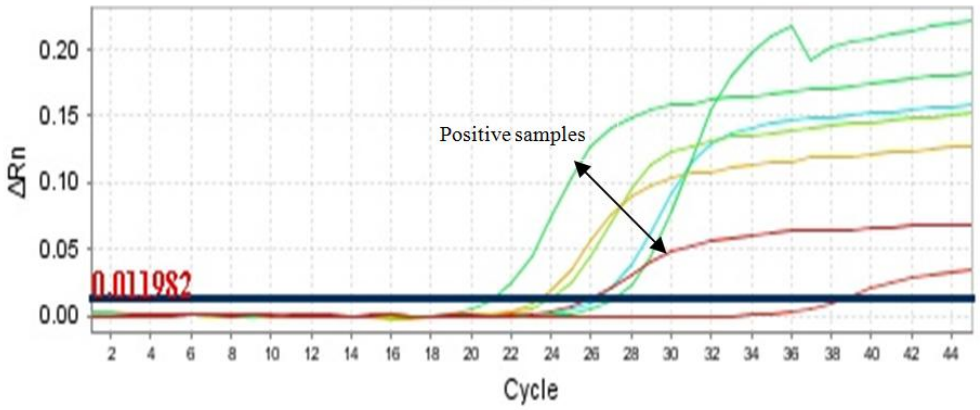

Fig. 3. PCR amplification of one step real-time RT-PCR for RSV positive samples in which that Ct values less than 30 and at the sametime that diagnosis with conventional RT-PCR

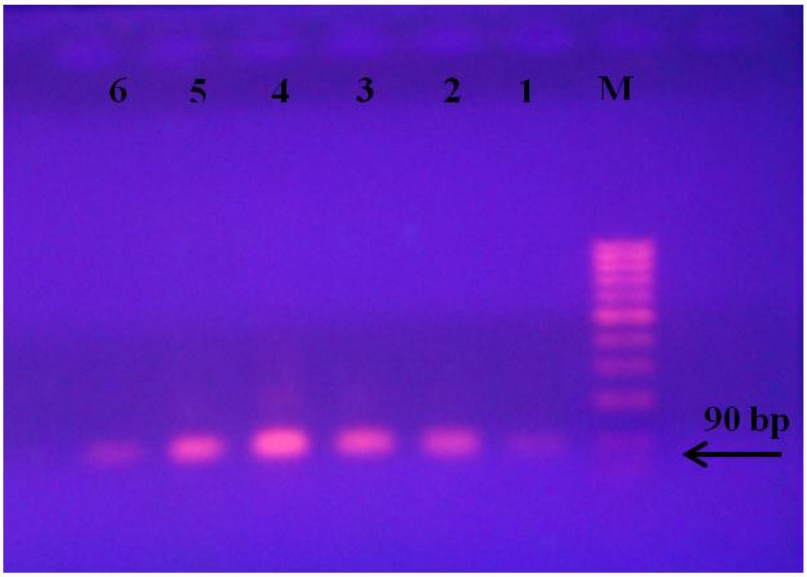

Fig. 4. Analysis of conventional RT-PCR sensitivity for RSV diagnosis in clinical swabs,Ct values of lanes 1 to 6 were 27.1, 23.94, 23.43, 21.03, 25.3 and 26.3, respectively

DISCUSSION

In the current study, RSV detection presented an important role along with influenza season among patient negatively for influenza virus who's shared clinical signs with flu-like illness. Thus, conventional and real-time RT-PCR assays used in this study for RSV detection. Additionally, the specificity of the used primers and probe was only with RSV RNA rather than other viruses like adenovirus, HSV, CMV, varicell-zoster, EBV, Coxsackie B3 and influenza viruses as designed by Mentel *et al*. (2003). Results showed that real-time RT-PCR had more ability to identify RNA-RSV infections because the

In Iraq, Odisho *et al*. 2010, whoreported that HRSV 5% in HEP-2 cell line culture from children with sever bronchitis and pneumonia. Using the cell culture assay for virus isolation and detection depended on viral infectivity which affected with many factors like timing factor; it was found that 90% reduction in virus titer within 2 hours (Falsey and Walsh, 2000). Meanwhile, real-time RT-PCR had a higher sensitivity than viral culture for detecting RSV (Van de Pol *et al*., 2010). Previous study comparing RT-PCR with virus isolation and antigen detection showed that RT-PCR assay increased frequency of RSV detection from 30% to 112% compared with

these two attributes assays (Erdman *et al*., 2003). Hence, real-time RT-PCR assay in the current study increased to viral identification due to it was detected the viral nucleic acids in clinical sample than an intact viral liability. Moreover, it has the ability to detect also a low level of virus in clinical samples (Guendin *et al*., 2003). Consequently, its improved patient management, infection containment and control to reduce morbidity and mortality among patient with a sever RSV infection (Dowell *et al*., 1996).The highest incidence of RSV infection (63.15%) in the winter period (December) was appeared, this was agreement with many investigators listed that RSV tends to occur in relation to the rainy season: however, in locations closer to the equator with perennial rain fall, RSV activity was almost continuous (Matheson *et al*., 2009). Therefore, RSV is an important pathogen contributing to the burden of influenza-like illness in the entire community in winter (Dowell *et al*., 1996).

RSV accounted for a higher percentage of the positive viral diagnosis in the youngest age-groups in two of three years, and was the most significant pathogen in children younger than 1 year (Zambon *et al*., 2001; Deraz *et al*., 2012). It infects over 90% of children by the age of two and cause cold-like symptoms that frequently progress to lower respiratory disease such as bronchitis and pneumonia (Nair *et al*., 2010), and resulting in significant morbidity, with mortality rates approximately 10-fold higher than for influenza in those ages (Thompson *et al*., 2003). Relatively, there is a small significant of RSV infection in children under two years old (P=0.04), within infected children in this study. Furthermore, most of that age group has been required higher viral load (low Ct) that associated with disease severity from those larger children with mild disease and lower viral load (high Ct).Other investigators found a highly significant linear relationship between the log of the input target nucleic acid copy number and Ct values, hence the Ct values indicate for concentration (viral load) of unknown quantities of RSV-RNA in a clinical sample (Borg *et al*., 2003). The results of this study showed positive correlation between low Ct value and infection severity, in spite of there is three cases of acute bronchitis had high Ct (low viral load). It is likely that the capability of the infant's immune response to reduce viral replication is the major determinant of RSV load, also the load decreased with longer durations of symptoms before specimen collection (De Vincenzo *et al*., 2005; ElSaleeby *et al*., 2011).Course of treatment in the early might reduce direct viral replication that may contribute to the low viral quantity in nasopharyngeal secretion (ElSaleeby *et al*., 2011). There are several issues complicated with RSV load including mucous secretions from the airways are non-homogenous, amount and composition of airway secretions over time are variable, and the lower respiratory tract is not easily accessible for sampling (Falsey *et al*., 2003).This study concludes a using multiplex PCR is important for diagnosis of RSV along with influenza virus, addition to human rhinovirus and the human metapneumonia virus as a potential infection of influenza like-illness which occur in the second most common cold pathogen.

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