Respiratory syncytial virus induces TARC and MDC in children who contracting from respiratory tract infection

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Abstract:

Background: Human RSV is belonged pneumovirus genus, pneumovirinea subfamily, with paramyxoviridae and order of Mononegavirales; it is the major etiological agent of respiratory tract illness particularly in children, and it provoke allergic and asthma exaggeration, so, the respiratory tract epithelial cell are stimulated and produce several chemokines such MDC and TARC that play a major role in asthma attach.

Material and method: Molecular techniques are performed in this study such as RT-PCR and gene expression study.

Results: Out of 230 children suffering from respiratory tract infection, 8 (8%) and 14 (14%) were detected with the respiratory syncytial virus type A and B respectively, and all epithelial cells of patients who have infected with RSV express TARC and MDC are essential role to have known severity and hyperresponsiveness of allergy.

Conclusion: Measurement of host gene expression such as TARC and MDC, which increased level in allergy such as asthma, particularly in childhood, that induced by RSV are essential role to have known severity and hyperresponsiveness of allergy.

Key words: RSV, TARC, MDC, RTI

Introduction

Human RSV is belonged pneumovirus genus, pneumovirinea subfamily, with paramyxoviridae and order of Mononegavirales, the virion of RSV is made up of enveloped lipid with asymmetrical spherical shaped, 150 to 300 nm in measurement. Other morphology of virion that are 60-100 nm and up to 10 nm in length, filament-like, could be seen in both infected cultures and preparation of the virus (Brown, 2004). The viral envelope is a lipid bilayer obtained from the host plasmatic layer,

Respiratory syncytial virus (RSV) is a ubiquitous infection that causes airway route aggravation, for example, laryngitis, croup and it likewise causes bronchitis. (Brown, 2002; Tripp 2005). The major cause of bronchiolitis in worldwide is RSV, which form up to 70-80% of all other lower respiratory tract illness (Henrikson, 2004). The most experienced children with RSV are symptomatic, and have a notable clinical picture, comparable with upper respiratory tract, the lower respiratory tract is more frequent, and the predominant sign is wheezing, the children who are in the first three months of life, have severely clinical signs (Ogra, 2004; Hall, 2012).

Respiratory syncytial virus infection in early life is associated with the succeeding initial allergic airway illness (Sigurs, 2000), newly, animal experimental practice have been used to additional inspect this relation, whither the infection with RSV, previously, during, or taking after allergen trial being displayed to modify allergic signs (Matsuse *et al*., 2000; Lukacs *et al*., 2001).

Chemokines are a superfamily of proteins can be organized into really diverse gatherings: three groups of small basic (heparin-binding) proteins, labeled the C, CC, and CXC chemokines (depend on the number and spacing of highly conserved NH_2-terminal cysteine residues), and a fourth, indirectly related group, the CX3C chemokines, made out of substantial, membrane- bound glycoproteins linked through a COOH-mucin-like area . Those receptors for the chemokines are express Edina cell sort limited design has permitted specificity in chemokine activity—for instance, individuals from the C group essentially stimulate lymphocyte chemotaxis, individuals from the CXC cluster affect neutrophil chemotaxis, and the CC group revitalizes monocyte,

lymphocyte, and eosinophil chemotaxis **(**Oppenheim *et al*., 1991**;** Baggiolini *et al*., 1997).

There are several CC chemokines network expression can be induced RSV infection, for instance, thymus and activation regulated chemokine (TARC), RANTES, MCP-1, macrophage-derived chemokine (MDC) and (MIP-1a and 1b), CXC, CX3C. However, TARC is a CC chemokine which is chemotactic for naïve CD4 T lymphocytes and immature dendritic cells (Lieberam *et al*.; 1999). The receptor CCR4 can be allowed to bind with CCL17/TARC and CCL22/ MDC. The CCR4 is expressed on several a subpopulation of peripheral blood such as mature dendritic cells, lymphocytes, thymocytes and on blood platelets (Lukacs, et al., 2003). The CCR4 expression associated with TH2 responses. Raised CCR4 levels can proceed up two days after induced by T-cell receptor (Vestergaard *et al*., 2003). Macrophage-derived chemokine (MDC/ CCL22) made up of 69 amino acid residues, synthesized by macrophage cells linage (Pinho *et al*., 2003), other cells are consider as a source of MDC production including monocyte-derived dendritic cells, natural killer (NK) cells and bronchial epithelial cells (Hirata *et al*., 2003), the MDC is clustered on chromosome 16q13, it contain of the 4 cysteine motif and highly conserved residues characteristic of CC chemokines, it is identity with other human chemokines such as TARC by less than 35%, and the later, TARC, is closed human comparative (Mantovani *et al*., 2000; Kakinuma *et al*., 2002).

Thymus-and activation-regulated chemokines (TARC) is a highly specific ligand of CCR4, and it is synthesized and produced by several cells including monocytes, dendritic cells and it as a serve assist recruitment, activation and development of Th2-polarized cells for CCR4 expression (Leung *et al*., 2002; Fujisawa *et al*., 2002).

Aims of study

- Identification of both human genetic groups of respiratory syncytial virus from children who have gone respiratory tract infection.
- Establish the relationship between RSV and allergy exaggeration through determination the host gene expression (TARC and MDC) which induced by RSV infection.

Material and methods

Data sets

Two Hundred and thirty children patients were randomly chosen, clinically, all patients were suffering from respiratory tract illness (RTI), the database of them were registered in this study, which encompass, name of the patient , age; gender and the major clinical symptoms of RTI, such as fever, cough, sneezing, nasal discharge (rhinorrhea),and asthma attach which evaluated, principally, by the consultant pediatricians via take the major clinical features of asthma which involved (wheezing and dyspnea), the age of elected patients was from several days to fourteen years old of both sexes, from Al- Sader Teaching Hospital at Al Amara city during the period from December 2014- April 2015. The information of each patient was taken based on the questionnaire format which has been constructed by the supervisors and pediatricians, as set out in the appendix.

Primers and Probes designing

The primers and probe were designed in this study by using the complete sequence of Nucleoprotein gene RSV-A (GenBank: KF973340.1), RSV-B (GenBank: KF893260.1), and hMPV (GenBank: KF891365.1) from NCBI-GenBank and Primer3 plus design. The primers were provided by (Bioneer/Korea) as the following table 1.

Table 1: Primers and probes that used in this study

Primer	Sequence		Amplicon
RSV-A primer	F	5′-TGCAGGGCAAGTGATGTTAC-3′	86bp
	R	5′-TTTCTGCTTGCACACTAGCG-3′	
RSV-A probe	5′-VIC-GGTGGGGAGTCTTAGCAAAATCAGTT-BHQ-1		
RSV-B primer	F	5′-TGTGCACTTTGGCATTGCAC-3′	101bp
	R	5′-TTACTTGCCCTGAACCATAGGC-3′	
RSV-B probe	NED-TCCACAAGAGGGGGTAGTAGAGTTGA-BHQ-1		
hMPV primer	F	5′-AGAAACTCAGGCAGTGAAGTCC-3′	130bp
	R	5′-TCTCTTCCACCCAGCTTTTCTC-3′	
hMPV probe	FAM- ACCAGAACGTACTCCTTGGGGAA-BHQ1		

F: Foreword, **R**: Reverse

Gene expression study primers

The gene expression primers of chemokines gene, which were measured from the airway epithelial cells that shaded with the NP swab, and hMPV genes were designed in this study by using NCBI-Genbank and Primer3 plus design Genbank codes: reference Actin gene (NM-001101.3), chemokine MDC gene (U83171.1), chemokine TARC gene (XM-011523256.1), M2-2 protein gene hMPV (AY530095.1), and G-protein gene hMPV (JQ309682.1), and these primers were provided by (Bioneer/ Korea), as following in the table 2.

Table 2: **The MDC, TARC G glycoprotein, M2-2, and Actin primers**

Primer	Sequence		Amplicon
Actin	F	5′-TCGTGCGTGACATTAAGGAG-3′	133bp
	R	5′-TTGCCAATGGTGATGACCTG-3′	
MDC	F	5′-TGTGAAGCCCCAAATTTGCC-3′	124bp
	R	5′-AAGCCAAGACCACACCATTG-3′	
TARC	F	5′-TGGGGCAATGTCAATGTTGG-3′	125bp
	R	5′-AGTTCTGTGTACCCAGCCAAG-3′	

F: Foreword**, R:** Reverse

Viral RNA Extraction

- Viral RNA was extracted from frozen nasopharyngeal swabs samples by using AccuZol^{TM} Total RNA extraction kit (Bioneer, Korea) and done according to company instructions as following steps:
- The two hundred microliter (200µl) of nasopharyngeal fluid samples was transferred by sterile pipette into sterilized and fresh 1.5ml Eppendorf tube, then 1ml AccuZolTM reagent an mixed by vortex.

- The two hundred microliter (200µl) of chloroform was added to each Eppendorf tube and shaken vigorously for 30 seconds.
- The mixture was incubated in ice for 5 minutes.
- Afterward, the mixture was centrifuged at 12,000 rpm, 4°C, for 15 minutes.
- The supernatant was transported to a new Eppendorf tube, and 500µl isopropanol was added.
- The mixture was mixed by inverting the tube for 4-5 times and incubated at 4°C for 10 minutes.
- The mixture was centrifuged at 12,000 rpm, 4°C, for 10 minutes.
- The supernatant was thrown away.
- The 80% of ethanol alcohol was added into each tube and well mixed by vortex, then centrifuged at 12,000 rpm, 4°C for 5 minutes.
- The supernatant was discarded and the RNA pellet left to dry at room air for 5 minutes.
- After that, DEPC water (50µl) was added to RNA pellet tubes and well mixed by vortex to solute the RNA pellet.
- The extracted RNA sample was checked by Nanodrop spectrophotometer, and then kept at -20°C freezers.

Estimation of extracted total RNA yield

The total extracted RNA was assessed by a Nanodrop spectrophotometer (THERMO. USA), There are two quality controls were performed on extracted RNA. First one is to determine the quantity of RNA (ng/µL), and the second is the purity of RNA by reading the absorbance in a spectrophotometer at 260 nm and 280 nm in same Nanodrop device as follow:

- After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, RNA).
- A dry wipe was taken and cleaned, the measurement pedestals several times. Then carefully pipetted 2µl of free nuclease water and put on the surface of the lower measurement pedestal to blanking of Nanodrop.
- After that, the pedestals are cleaned and pipette 1µl of RNA sample for measurement.

Reverse Transcription cDNA synthesis step

The total RNA samples were used in cDNA synthesis step by using AccuPower® RocketScript RT PreMix kit that delivered from Bioneer Company, Korea and done according to company instructions as following in the table 3

Table 3: The RT master mix subjects

RT master mix	Volume
Total RNA 100ng/ul	10µl
Random Hexamer primer (50 pmol)	1µl
DEPC water	9µl
Total	20µl

This RT PreMix was placed in AccuPower RocketScript RT PreMix tubes that contains lyophilized Reverse transcriptase enzyme and then dissolved completely by vortex and briefly spinning down.

The RNA transformed into cDNA in thermocycler under the following thermocycler conditions, see table 4.

Table 4: Steps of convert of RNA into cDNA

Step	Temperature	Time
cDNA synthesis (RT step)	50 °C	1 hour
Heat inactivation	95 °C	5 minutes

Real-Time PCR (qPCR)

The qPCR was performed for detection of respiratory syncytial virus type A (RSV-A), respiratory syncytial virus type B (RSV-B) and Human metapneumonia virus (hMPV) based on nucleoprotein gene and this technique was carried out according to method described by Do LA *et al*., (2011) as following steps:

Reverse Transcription Real-Time PCR (RT-PCR)

One step RT-qPCR was performed for detection of respiratory syncytial virus type A (RSV-A), respiratory syncytial virus type B (RSV-B) and Human metapneumonia virus (hMPV) based on nucleoprotein gene and this technique was carried out according to method pronounced in GoTaq® 1-Step RT-qPCR manual technique System.

Real-Time PCR master mix preparation

Real-Time PCR master mix was prepared by using one step Reverse Transcription and Real-Time PCR detection kit (AccuPower RocketScript RT-qPCR PreMix, Bioneer. Korea), and done according to the company instructions as in the following table 5.

Table 5: The RT-PCR mastermix constituents:

RT-PCR mastermix	**Volume**
Total RNA template	5µL
RSV-A, RSV-B, hMPV Forward primer (20pmol)	2.5µL
RSV-A, RSV-B, hMPV Reverse primer (20pmol)	2.5µL
RSV-A, RSV-B, hMPVprobe (25pmol)	2.5µL
DEPC water	37.5 µL
Total	50µL

The RT-PCR master mix reaction components that mentioned in table (5) above were added into RT-PCR tube containing (8 wells strips tubes which containing RocketScript reverse transcriptase and TaqMan probe premix). Then all strips tubes vortexing for mixing the components and centrifuged for 3000rpm for 3 minutes in Exispin centrifuge, after that transferred into Real-Time PCR thermocycler.

Real-Time PCR Thermocycler conditions

Real-Time PCR thermocycler conditions was set based on to primer toughening temperature and RT-PCR TaqMan kit instructions as in the following table 6.

Table 6: The RT-PCR thermocycler conditions

Step	Condition	Cycle
Reverse transcriptase	50 °C, 15 min	1
Pre-Denaturation	95 °C, 5 min	1
Denaturation	95 °C, 20 sec	50
Annealing/Extension	60 °C, 30 sec	
Detection (Scan)		

Real-Time PCR Thermocycler conditions

A real-Time PCR thermocycler condition was set according to primer annealing temperature and qPCR TaqMan kit instructions as in the following table7:

Table 7: qPCR thermocycler conditions

Step	Condition	Cycle
Pre-Denaturation	95 °C 5 min	1
Denaturation	95 °C 20 sec	50
Annealing/Extension	60 °C 30 sec	
Detection (Scan)		

Real-Time PCR Data analysis

The quantitative PCR (qPCR) data analysis was performed by calculation the threshold cycle number (CT value) that the positive amplification in Real-Time PCR cycle number.

The DNA sequencing study

The DNA sequencing was performed for genotyping of positive Human metapneumovirus by qPCR technique. The DNA sequencing method was based on hMPV fusion protein gene primers that done in PCR technique as following steps:

1- Preparation of the PCR mastermix

The PCR master mix was prepared by using (AccuPower PCR PreMix Kit) and this master mix done according to company instructions as in following table 8.

Table 8: The PCR master mix of gene expression contents

PCR Master mix	Volume
DNA template	5 µL
F gene Forward primer,(10pmol)	1.5µL
F gene Reveres primer,(10pmol)	1.5 µL
PCR water	12 µL
Total volume	20 µL

After that, these PCR master mix component that mentioned in table (2-13) placed in standard AccuPower PCR PreMix Kit that encompassing all other components which needed to PCR reaction such as (*Taq* DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$, stabilizer, and tracking dye).Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes, and then placed in PCR Thermocycler (MyGene. Korea).

2- The conditions of the PCR Thermocycler

The PCR thermocycler conditions were done by using conventional PCR thermocycler system as following table 9

Table 9: The PCR thermocycler condition of gene expression

PCR step	Temp.	Time	repeat
Initial Denaturation	95°C	5min	1
Denaturation	95°C	30sec.	30 cycle
Annealing	58°C	30sec	
Extension	72°C	1min	
Final extension	72°C	5min	1
Hold	4°C	Repeatedly	-

3- DNA sequencing method

The sequencing of the PCR product for hMPV F gene (428bp) was purified from agarose gel by using (EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic. Canada). As the following steps:

- The specific PCR product was removed from the gel by clean, sharp scalpel. And then, transferred into a 1.5mL microcentrifuge tube.
- The four hundred microliter (400µl) of binding buffer II was added to gel fragment, then, incubated at 60ºC for 10 minutes and mixed until the agarose gel is completely dissolved.
- Add the above mixture to the EZ-10 column and let stand for 2 minutes. Centrifuge at 10,000 rpm for 2 minutes and discard the flow-through in the tube.
- The seven hundred and fifty microliter (750µl) of washing solution was added to each tube and centrifuged at 10000 rpm for one minute. Then, solution discarded.
- After that, the step 4 was repeated and centrifuged at 10000rpm for an additional minute to remove any residual wash Buffer.

- The column was placed in a clean 1.5ml microcentrifuge tube and added 30µl of Elution Buffer to the center of the column and incubated at room temperature for 2 minutes, then, the tube was centrifuged at 10000 rpm for 2 minutes to elute PCR product and store at -20ºC.

After that, the purified GIF gene PCR product samples were directed to Bioneer Company in Korea for achieved the DNA sequencing by AB DNA sequencing system.

Phylogenetic study was done based on NCBI-Blast alignment documentation and Neighbor Distance Phylogenetic tree analysis (Mega ver. 6).

Gene expression study

The gene expression study was performed for estimation of relative gene expression of some chemokine genes (MDC gene) and (TARC gene) which related to human respiratory syncytial virus (hRSV) in only cDNA template of positive samples by using Real-Time PCR based Syber Green dye amplification. This method was carried out according to method described by Zhang *et al*., (2001) as following steps:

1- Real-Time PCR (qPCR) mastermix preparation

Quantitative PCR mastermix was prepared by using AccuPower™ Green Star Real-Time PCR kit based SYBER Green dye detection of gene amplification in Real-Time PCR system and include in the following 10.

Table 10: The Real-Time PCR (qPCR) mastermix preparation of gene expression

The qPCR mastermix	Volume
cDNA template (100ng)	3µL
Target genes or reference gene Forward primer(10 pmol)	1 µL
Target genes or reference gene Reverse primer (10 pmol)	1 µL
DEPC water	15 µL
Total	20 µL

After that, these qPCR mastermix component that mentioned above AccuPower Green star qPCR premix standard plate tubes that have the Syber green dye and other PCR extension constituents, then the plate mixed by Exispin vortex centrifuge for 3 minutes, then placed in Miniopticon Real-Time PCR system .

After that, the qPCR plate was loaded and the following thermocycler protocol in the following table 11.

Table 11: Thermocycler protocol of qPCR of gene expression

The qPCR steps	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	5min	1
Denaturation	95 °C	15 sec	45
Annealing\Extension Detection(scan)	60 °C	30 sec	
Melting	60-95°C	0.5 sec	1

Data analysis of qRT-PCR

The data results of qRT-PCR for target and housekeeping genes were analyzed by the relative quantification gene expression levels (fold change) ΔCT Reference technique that designated by Livak and Schmittgen, (2001). The relative quantification method, quantities obtained from q RT-PCR experiment must be normalized in such a way that the data become biologically meaningful. In this process, one of the investigational samples is the calibrator such as (Control samples) each of the normalized target values (CT values) is separated by the calibrator normalized target value to generate the relative expression levels. After that, the ΔCT Way with a Reference Gene was used as following equations:

Relative expression formula:

Ratio (reference/target) = 2CT (reference) – CT (target).

Table (2-17): The ΔCT Method for estimation the relative gene expression.

Gene	Test (treatment, group)	Cal. (control, group)
Target gene	CT (target, test)	CT (target, cal.)
Reference	CT (ref, test)	CT (ref., cal.)

First, normalize the CT of the reference (ref) gene to that of the target gene, for calibrator sample:

ΔCT (calibrator) = CT (ref., calibrator) - CT (target, calibrator)

Second, normalize the CT of the reference (ref) gene to that of the target gene, for test sample:

ΔCT (test) = CT (ref., test) - CT (target, test)

ΔΔCT= ΔCT (test) - ΔCT (calibrator)

Fold change = $2^{-\Delta\Delta CT}$

Ratio (reference/target) = 2CT (reference)-CT (target)

So, the relative expression was separated by the expression value of chosen calibrator for each expression ratio of the test sample.

The Real-Time PCR Data analysis

RT-PCR data analysis was performed by calculation the threshold cycle number (CT value) that presented the positive amplification in Real-Time PCR cycle number.

Results

The following data involved the display certain chemokines (CC) which induced by RSV and their role in pulmonary diseases such as allergy and asthma exaggeration, these CC are represented with thymus-and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC), each those CC is expressed on bronchial epithelial cells and alveolar macrophage, they have common receptor which is chemokines receptors-4 (CCR4) that selectively expressed on Th2 cells which play a crucial role in asthma development. Whatever, the TRAC and MDC are also elevated in other chronic diseases such as atherosclerosis, Crohn's disease, and cigarette smoke-induced acute and chronic airway inflammation (Jugde *et al*., 2001), but these diseases are beyond the scope of the current study. However, we will present all tables and their figures (positive RSV) and (negative RSV as a control), and then the results are discussed.

Interpretation results of RSV-A&B TARC and MDC gene expression

In the below tables show the expression level of TRAC and MDC gene, which induced by both types of RSV (A and B), all these tables of positive RSV (A and B) have shown a high level fold change expression, and compared to the negative-RSV-A patients which represented in the following figures (1), there is a significantly different, the p-value is (0.0001), it is detonating RSV-A, B have induced the infected bronchial epithelial cells and alveolar macrophage to produce TRAC and MDC that ligand the specific receptor on the Th2, CCR4,. Th2 cells have an essential role in allergic diseases like asthma and allergic rhinitis (Panina-Bordignon *et al*., 2001; Berin, 2002; Leung *et al*., 2004). Several studies have convinced the association between

RSV and asthma development, as the study that worked by Zhang *et al*., (2001), they have concluded through their experiment, that RSV has a potent inducer to produce several chemokines such as TARC, RANTES, MCP-1, and MDC, and then they explained their result which encompassed the importance of TARC and MDC in asthma development and exaggeration, TARC an effective chemotactic for CD_4^+ T lymphocytes and immature dendritic cells, RSV-infected A549 cells can be induced for TARC production which has implicated in atopic asthma in human. In addition, other studies such as that achieved by Monick *et al*., (2007), they proved RSV induce Th2 cytokines such as IL-4 and IL-13 that improve production of TRAC; also they observed the IL-4 alone did produce very small amount of TRAC mRNA. In one study that done by Takeuchi *et al*., (2005) they have determined that TARC is a considering target for immunotherapy (IT) which has been observed the reduction amount of TRAC in patients who have suffered from allergic rhinitis after IT demonstration. MDC is another potent chemotactic, it has the capability to recruit Th2 lymphocytes, monocytes, immature DC, and IL-2-activated NK cells (Chantry *et al*., 1999). MDC is, as well, recognized in normal bronchial epithelial cells in human atopic asthma (Sekiya *et al*., 2000). The study like that done by Gonzalo *et al*., (1999) they have reported role of MDC which is essential chemokine in allergic ovalbumin-induced pulmonary inflammation in mice, MDC is neutralized by antibodies that lead to block acidophil recruitment. Furthermore, in original article that published by Egypt Journal Pediatric Allergy in 2005 which have been done by Mohammad H. Ezzat and his collogue, Kareem Y., they have measured MDC (CCL22) and its receptor CCR4 on peripheral blood T lymphocytes of asthmatic children, they founded the level of MDC was significantly higher in asthmatic children (Mohamed and Karim, 2005).

However, the MDC might recruit Th2 lymphocytes into airway of asthmatic case, Th2 lymphocytes intensify the allergic inflammatory reaction through IL-4, IL-5 and IL-13 production which provoke the B lymphocytes into plasma cells which responsible for IgE production, moreover the IL-5 stimulate eosinophils for activation and differentiation (Ryan, 1997; Brombacher, 2000).

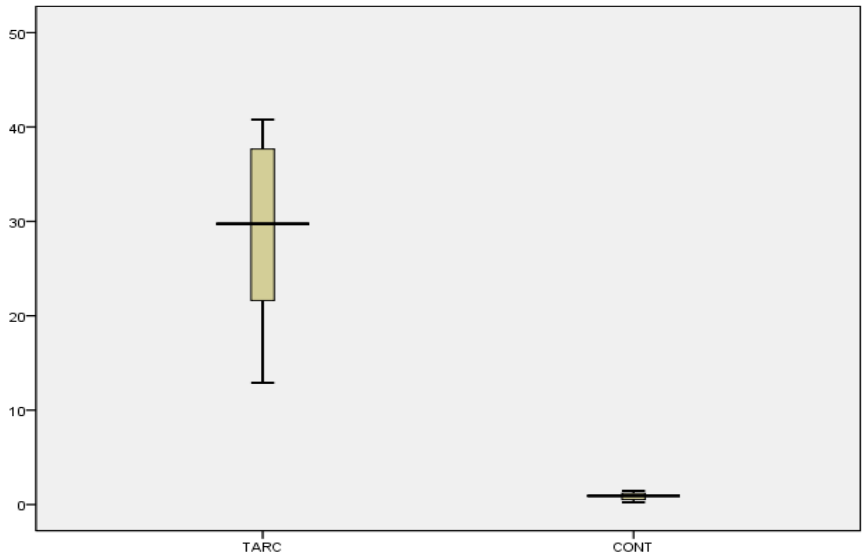

Figure 1: Boxplot shows comparison between RSV-A-induced TARC with control (RSV-A negative).

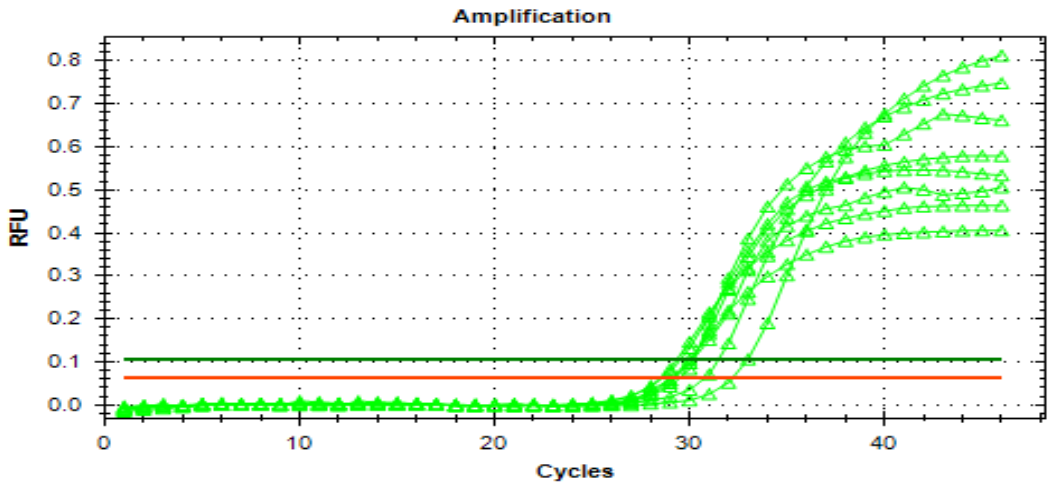

Figure 2: Real-Time PCR amplification plot for TRAC gene in Respiratory Syncytial Virus type A (RSV-A) positive patient samples.

In the figure (2) shows the expression level of TRAC gene, which induced by RSV-A that ranging from the high level 40.786 to lower level 19.160 folds.

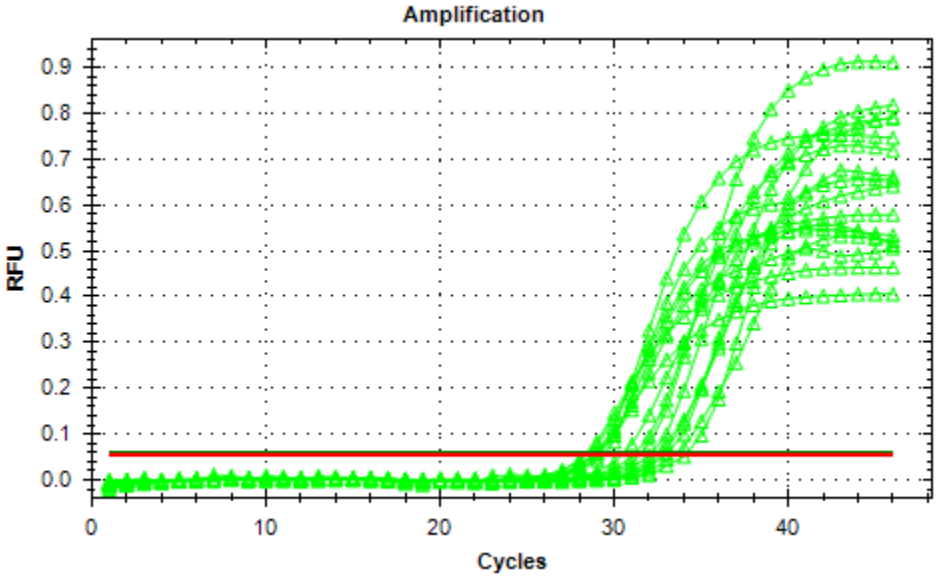

Figure 3: Real-Time PCR amplification plot for TRAC gene in Respiratory Syncytial Virus type B (RSV-B) positive patient sample

In the figure (3) shows the expression level of TRAC gene, which induced by RSV-B that ranging from the high level 64.000 to lower level 9.646 folds.

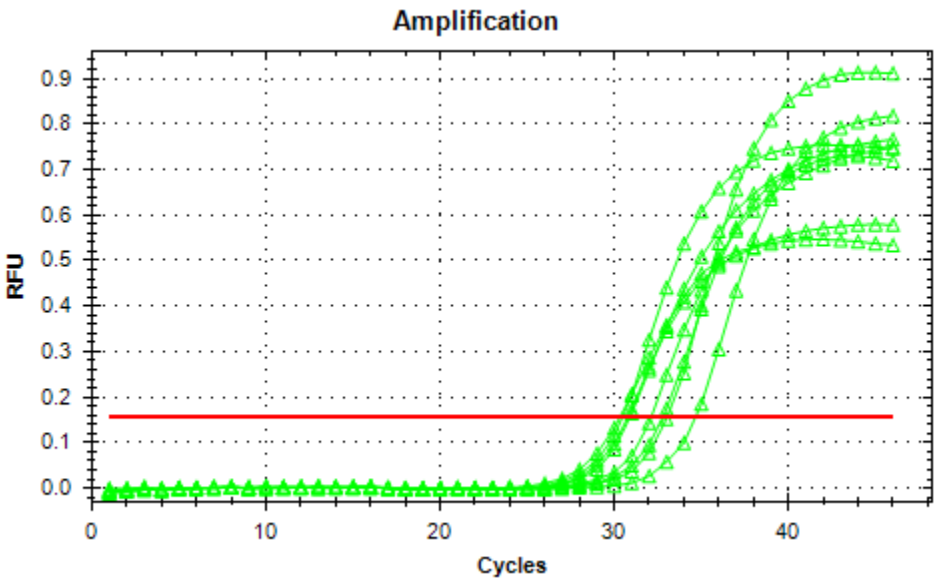

Figure 4: Real-Time PCR amplification plot for MDC gene in Respiratory Syncytial Virus type A (RSV-A) positive patient samples.

In the figure 4 shows the expression level of MDC gene, which induced by RSV-A that ranging from the high level 37.792 to lower level 7.727 folds.

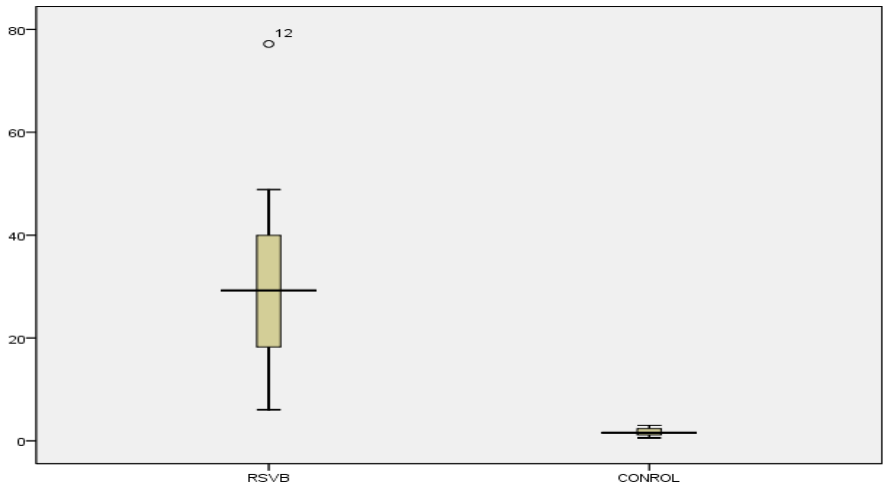

Figure 5: Bar box of RSV-B induced MDC and control (RSV-B negative)

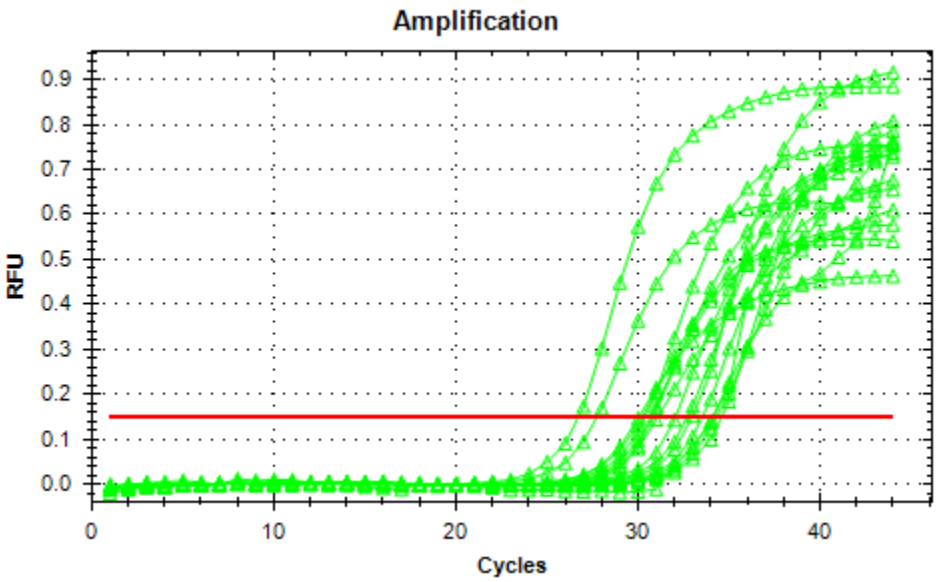

Figure 5: Real-Time PCR amplification plot for MDC gene in Respiratory Syncytial Virus type B (RSV-B) positive patient samples

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