Detection of Human Respiratory Syncytial Virus Associated with Asthmatic Patients Using Tissue culture and Real time – Polymerase Chain Reaction*.

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Alaa H. Al-Charrakh[1] **Ghanim A. Al-Mola**[2] **Jalal A. T. Al-Azzawi**[1]
[1] Microbiology department, College of Medicine, Babylon University, Babil Governorate, Iraq.
[2] Biology department, College of Science for Women, Babylon University, Babil Governorate, Iraq.
e.mail: j.tofah@yahoo.com

Abstract: This study aimed to determine incidence human respiratory syncytial virus (hRSV) isolated from patients with asthma in Wasit province, Iraq. Blood samples were collected for measurement total and differential white blood cells (WBCs). Results showed total of WBCs and differential were significant ($P<0.001$) correlation in neutrophils, lymphocytes, eosinophils and basophils counts among different groups studied. Enzyme linked immunosorbent assay (ELISA) technique has been applied for detection of total-IgE antibodies. Results revealed that the highest total-IgE antibodies titer in sera were significantly difference ($p < 0.01$).. A total of 80 nasopharyngeal swabs were immediately dipped in transport media and stored until using for the detection of suspected hRSV patients group were used in real time – polymerase chain reaction (RT-PCR) from which 15 samples (18.75 %) out of 80 samples gave positive result for this test. The isolated hRSV 9 samples (11.25%) was cultured successfully on HeLa cell line, Cervical human carcinoma, as there were characteristic cytopathic effects observed in monolayer of cells line. All positive samples in tissue culture were confirmed by RT-PCR which indicated that the samples were positive for hRSV indicate cultured of it. The present of the virus in patients with asthma.

Biology Classification QP_1 -345

keyword: human respiratory syncytial virus, asthma, total-IgE, cultured on HeLa cell line.

Introduction

Asthma exacerbation have been shown to be a major cause of morbidity and mortality and up to 80% of the exacerbations are linked to viral infections(1). Asthma is characterized by acute episodes of airway obstruction precipitated by respiratory infection and the release of IgE depended mediators, airway inflammation resulting from an inappropriate response to either infectious or allergic antigens is a finding common to the different manifestations of asthma (2).

Asthmatics with viral infection but no sensitization show lower rates of hospital admission (3). This effect is due to synergism between allergens and viruses. When RSV infects bronchial cells, the bronchial cells produce various cytokines and chemokines. These responses cause hyperresponsiveness in bronchial cells. In other words, RSV infection might create a preparatory step as the first step in the development of asthma (4).

Evidence for the importance of viral respiratory infections in the development of asthma comes from studies indicating that sever paramyxoviral infections early in life impart a markedly increased risk for asthma later in childhood. RSV is a paramyxovirus that infects nearly all children by the age of 2 years (5). Although most of these infections have no known sequelae, infants requiring hospitalization for severe RSV infection in the first 6 months of life have a nearly 8-fold increase risk of developing asthma (6).

Kumhni and Silverman, 2009 thought that associated between RSV and asthma is due to shared predisposition rather than to a causal effect of RSV. Respiratory viruses are detected in the majority of asthma exacerbations in both children 80 – 85% and adults 75 – 80% (7).

The infections caused by RSV ranging from acute upper respiratory tract infections to sever acute lower respiratory tract infections like bronchiolitis and pneumonia (8).

The aim of this study was to determine the incidence of Respiratory syncytial virus diagnostic from nasopharyngeal swabs associated with asthmatic patients in the Wasit province, Iraq.

Materials and Methods:

Study Subjects

This study included Two groups of subjects:

A- A total of 80 specimens were collected from patients suffering from exacerbation asthma who were admitted to Al-Karrama'a Teaching Hospital and Al-Zahra'a General Teaching Hospital in Wasit province / Iraq during the period from January 2013 to May 2013. The patients age were ranged from 1 – 15 years. Two specimens were taken from each patient, nasopharyngeal swab and 3 – 5 ml of freshly drawn venous blood.

Nasopharyngeal swabs were collected by inserting a dry calcium alginate, aluminum-shafted swab into the nasopharyngeal area. The swab was allowed to remain in the area for 10 – 30 seconds and then rotated and withdrawn. One swab from each patient was place in 3 ml transport medium, vircell, and the second swab put in 2 ml transport medium, vircell, that put in an ice bag until be taken to the laboratory for real time PCR and fluorescent assay respectively, and store at -80 $^{\circ}$C for other time.

Blood samples 3 ml of venous blood was drowning from each patients. Blood samples were divided into 2 tubes; the first with 1 ml containing EDTA to prevent clotting were used for measuring white blood cell total and differential white blood cells. The second, gel tube with 2 ml which was left to clot and separation of serum by centrifugation at 3000 r.p.m. (9) for 10 minutes, after sera samples were carefully transferred to eppendorf tubes and store at –20°C until use.

B- Twenty specimens were collected from apparently healthy control group, who had no history of asthma.

- **Statistical Analysis**

All results were performed by statistical tests. Normally distributed data were expressed as mean ± SD. Difference between the groups examined using the t-test and a p-value of ≤0.05 was taken as statistiacally significant.

Results and Discussion

Asthma Distribution by Age and gender:

The demographical distribution of the studied groups according to the age were shown in table 1. The results clarified that the age was ranged between < 1 – 15 years and the mean ± SE for asthmatic patients was 4.768 ± 3.180.

Table 1: Distribution of patients according to age.

Age (year)	Patients group No. (%)	Control group No. (%)
5≥	62 (77.5%)	14 (70%)
6 – 10	13 (16.25%)	4 (20%)
11 – 15	5 (6.25%)	2 (10%)
Total	**80**	**20**

The monthly distribution of cases showed that highest frequency was during February and January 21 and 19 out of 80 patients 26.25 and 23.75 %, respectively, Table 2. There were differences between the numbers of patients in varying months of year, whereas the winter months like January, February had a higher rate of patients consulted the May month had less number, this because of increase in the prevalence of respiratory disorder induce asthma exacerbation in winter months than in other months or season for example July (10). The result was matched with that recorded by (11) in Iraq, who mentioned that distribution rate was higher in January and February in Iraq and (12) in USA who mention at winter virus peak had an increased risk of bronchiolitis in infancy and of asthma during childhood.

Table 2: The monthly distribution of asthma exacerbation cases.

Month	Patients group No. (%)	Control group No. (%)
January	19 (23.75 %)	4 (20%)
February	21 (26.25 %)	4 (20%)
March	16 (20 %)	4 (20%)
April	13 (16.25 %)	4 (20%)
May	11 (13.75 %)	4 (20%)
Total	**80**	**20**

Determination of WBCs total and differentials rate

Table 3 shows the mean level of white blood cell count was 10.659 ± 2.339 and 6.915 ± 0.831 in asthmatic patients and control groups respectively. This study showed that there was a significant difference in the total WBCs among different study groups using t-test (P< 0.001). The present results were almost similar to those obtained by Darwesh, (13) and Bicer *et al.,* (14). While there was a significant by using t-test (P < 0.001) correlation in neutrophils, lymphocytes, eosinophils and basophils counts among different groups studied. The role of viral infection in developing acute exacerbation of asthma is continuing to be defined. Subjects with asthma group had

significantly increased. The present results were almost similar to those obtained by Darwesh, (13) and Al-Watify and Al-Joubori, (15).The increase lymphocytes in peripheral blood of patients in present study refers to the role of these cells in viral and allergic infection. It is well document that lymphocytes are important part in the defense against viral infection. This possibly repeated or recurrent infection. This had been documented by many researched Itazawa *et al.* (16). Eosinophils and Basophils play a major role in allergic reactions. It contains a high affinity receptor, FcεR1 and are capable of an immediate response to allergen (17). Caughey *et al.* (18) in their study showed the basophils increase in asthma patients. Monocytes there were a non - significant correlation between asthmatic patients and control groups. The present results were almost similar to those obtained by Alaa and Thanaa, (19).

Table 3: Total and differential WBCs count of asthmatic patients and control groups

Parameter mean ± SD	patients group	Control group
WBCs x 10^3 cell/mm^3	10.659 ± 2.339 **A**	6.915 ± 0.831 **B**
NEU x 10^3 cell/mm^3	5.985 ± 1.277 **A**	4.431 ± 0.735 **B**
LYM x 10^3 cell/mm^3	3.064 ± 0.556 **A**	1.877 ± 0.213 **B**
MONO x 10^3 cell/mm^3	0.511 ± 0.432 **A**	0.46 ± 0.169 **A**
EOS x 10^3 cell/mm^3	1.008 ± 0.381 **A**	0.102 ± 0.094 **B**
BAS x 10^3 cell/mm^3	0.222 ± 0.163 **A**	0.044 ± 0.013 **B**
*** The same letter in one row means that there is no significant difference between these value**		

Determination of Total IgE

Results were presented in table 4 shows that IgE concentration are significantly ($p<0.001$) in asthmatic patients group have an expected IgE concentration 32.113 ± 6.676, 46.733 ± 19.474 and 90.484 ± 22.162 compared with control group 8.724 ± 9.957, 15.847 ± 8.423 and 14.472 ± 5.570 respectively, the distribution of IgE concentration according to the age group. This results consistent with study of Tavakkol *et al.* (20); where found a high significant differences in concentrations of IgE in patients compared with healthy individuals. Satwani *et al.*(21) showed eosinophilia along with raised serum IgE level to be a significant allergic marker. Several studies have reported the elevated of total serum IgE in asthmatics (12, 22 and 23). Therefore, it is in according with the well known fact that IgE plays a central role in the pathophysiology of allergic disorder such as asthma

Table 4: Concentration of IgE level IU/ml for asthma patients and controls.

Age group (years)	Group	IgE level IU/ml
< 1 – 2	**Asthmatic patients**	32.113 ± 6.676 **A**
	Control	8.724 ± 9.957 **B**
3 – 5	**Asthmatic patients**	46.733 ± 19.474 **A**
	Control	15.847 ± 8.423 **B**
6 – 15	**Asthmatic patients**	90.484 ± 22.162 **A**
	Control	14.472 ± 5.570 **B**
* The same letter in one age group means that there is no significant difference between these value		

Detection of hRSV by Real Time – PCR

A total of 80 nasopharyngeal swabs of suspected human respiratory syncytial virus infected asthmatic patients were used in real time – polymerase chain reaction, RT-PCR, from which 15 samples (18.75 %) give positive result for this test as illustrated in table 5 and figure 1.

Table 5 : Diagnosis of hRSV by Real time PCR

		HRSV	
Group	**Number of cases**	**positive cases**	**Negative cases**
Asthmatic patients	80	15 (18.75%)	65 (81.25%)
Control	20	0 (0%)	20 (100%)

Many respiratory infections caused by bacteria or viruses often share clinical features and symptoms which are difficult to distinguish clinically (24). Therefore, detection of these agents require to sensitive and effective method to give correct treatment and avoiding the unnecessary use of antibiotic. RT-PCR has been shown to be a better test for diagnosis than conventional assays and real-time PCR significantly reduces time to give results and has an advantage over conventional PCR as detection is dose in closed system in real time and decrease the risk of contamination (25).

If a non-immunocompromised child is RSV-positive by RT-PCR, it means that this child is acutely infected with RSV, has been ill recently with RSV, or is about to become ill with RSV. Almost all children are negative by RT-PCR after 12 – 21 days; but occasionally a child will remain positive for up to 4 weeks. During these longer periods of shedding detected by RT-PCR, the change increases that another undetected viral infection may be present, especially among young children have frequent viral infections during the respiratory season. Because of the small amount of viral antigen usually present in nasopharngeal aspirates collected from RSV-infected, current antigen detection assay may lack sufficient sensitivity to detect and diagnose RSV (26).

The results of RT-PCR was agreed with Jartti *et al*. (27) and Sung *et al.* (28), who had reported that percentages of infection were 18% and 8.4% respectively. Brice *et al.* (14) concluded that the RSV infection in RT-PCR 32%. The present results were almost similar to those obtained by Shameran and Al-Mola (29); Ali *et al.,* (30), in Iraq who found that RSV infection rates were 24% and 20%, respectively.

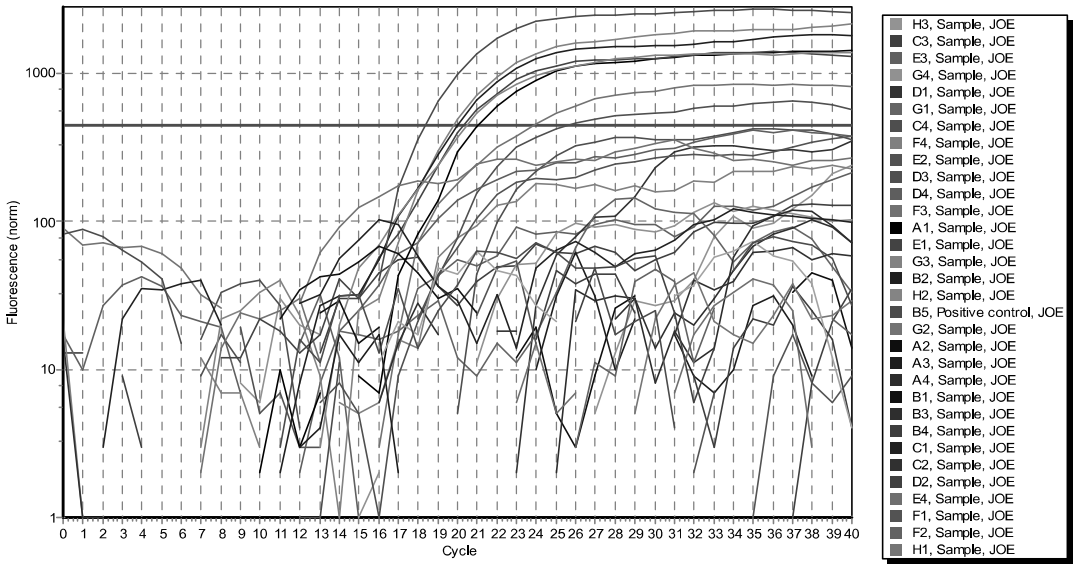


Figure 1: Real-Time PCR amplification log plot of human respiratory Syncytial virus (hRSV) from nasopharyngeal swabs samples. where, the positive control was appeared at (19.08 CT: Threshold cycle number), Internal positive control which appeared at (20.84 CT: Threshold cycle number), and the tested samples was positive reaction at (21.04 to 26.95 CT: Threshold cycle number), whereas, the negative control samples was appeared no amplification under Threshold cycle number.

Virus isolation in tissue culture

Isolation of hRSV was successfully done in HeLa cell line, Cervical human carcinoma, with 9 samples out of 80 samples (11.25%) table 6 as there were characteristic cytopathic effects observed in monolayer of HeLa cells line, ≥ 90% confluent after incubation more than 4 – 5 days at 37 ºC in the humidified CO_2 incubator.

Table 6: Diagnosis of hRSV by Tissue culture

		HRSV	
Group	**Number of cases**	**positive cases**	**Negative cases**
Asthmatic patients	80	9 (11.25%)	81 (88.75%)
Control	20	0 (0%)	20 (100%)

The main characteristic of cytopathic effects on HeLa cells was firstly observed at 3th day post infection in the first passage was cytoplasmic granulation, rounding and sloughing of the cells, followed by formation of syncytial cells and large empty plaques after two days, after that the dead cells began to detach from flask surface and float media. The control flasks remained unchanged, and showed no cytopathic effects as shown in figure 2, while the cytopathic effects is generalized to involve large areas of monolayer sheet two days later as shown in figure 3. In a study different response patterns were observed, with RSV infection of primary culture causing distinct peaks of viral replication and matched cytotoxic responses (31; 32). After ≤ 80% cytopathic appearance figure 3.8, infected cell cultures were frozen at – 80 ºC for further passages. All positive samples in tissue culture was confirmative by RT-PCR again which gave positive result that indicated to the infected by RSV.

This finding shows that the rate of identification of viral infection in human by viral culture is lower than that detected by RT-PCR, this result is agree with a study of Odisho *et al.*,(31); Ali *et al.,* (33), 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, can only be documented by measuring viral gene expression and genome replication in infected individuals and laboratory animals by PCR (34).

Overall, 15 (18.75%) of the 80 samples were positive by RT-PCR versus 9 (11.25%) by tissue culture table 7.

This study investigated the use of two nonculture and one culture methods of RSV isolation. A comparison of the sensitivity and sensitivity for the detection methods mentioned above is presented in table 8. This study appears increase the sensitivity of culture methods, HeLa cells. The cells were used before they reached a complete monolayer, thus increasing the change of observing the cytoplasmic effect of the RSV syncytia (35). Both of the rapid tests evaluated in the present report performed well as diagnostic tests in our laboratory and provide cost-effective alternative to tissue culture. Both particular, uses standard rapid techniques and equipment and does not require expertise in virology. More widespread availability of rapid RSV diagnostic tests

will hopefully result in early and appropriate use of antiviral therapy in patients at risk for serious RSV infection. This result was agreed with Lam *et al.,* (36); Bharaj *et al.,* (37), they are found the higher sensitivity and specificity in tissue culture than other tests such as RT-PCR.

Table 7: Tissue culture according to RT-PCR in detection of hRSV

Test		RT-PCR		
		Positive	Negative	Total
Tissue culture	Positive	9	0	9
	Negative	6	65	71
Total		15	65	80

Table 8: Sensitivity and specificity of tissue culture according RT-PCR in detection of hRSV.

Test compared	% Sensitivity	% Specificity
Culture vs RT-PCR	100%	91.56%

Sensitivity = $true\,positive \div (true\,positive + false\,negative) \times 100$.

The sensitivity of RT-PCR vs DFA is 69.23 %.

Specificity = $true\,negative \div (true\,negative + false\,positive) \times 100$.

The specificity of RT-PCR vs DFA is 60.33 %.

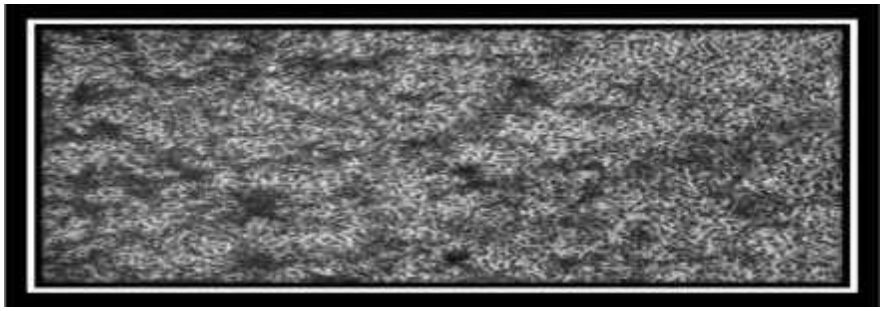
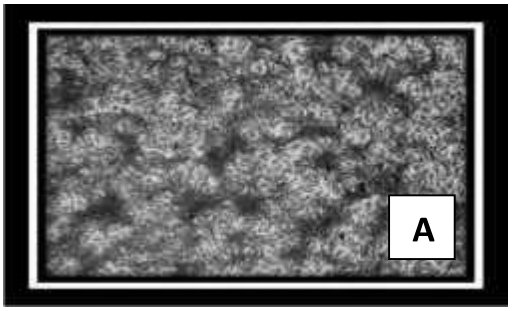
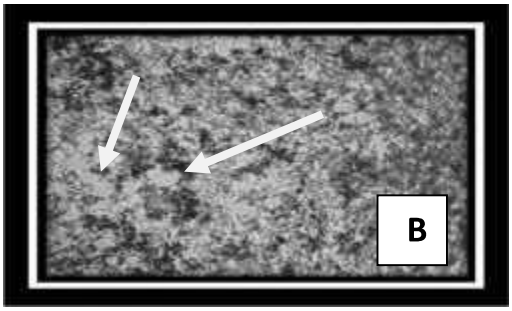


Figure 2: Uninfected HeLa cells line, (control negative) X200.





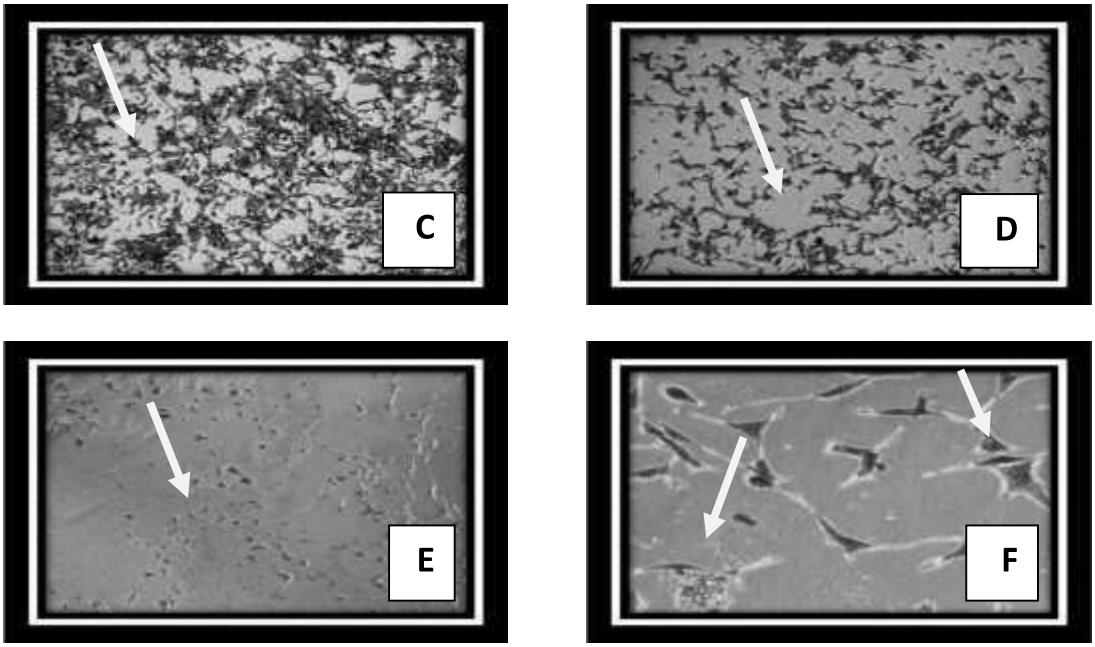


Figure 3: Cytopathic effect of RSV infection on Hella cells: A-Infected HeLa cells after 1 day. B- after 2 days. C- after 3 days. D- after 4 days. E- after 5 days x200. F- syncytia or giant cells formation, x400**.**

Virus titration

Virus infectivity was assayed by titration in microtiter plates with confluent monolayer of HeLa cells. Uninfected cells will be stained and the plaques will then appear as small clear areas figure 4. (approximately 0.5 – 3 mm in diameter against a white background) (38; 39).

According to virus purification for virus plaque assay protocol Count the plaques on each well and determine the virus titer as follows equation:

$$\text{PFU/ml} = \frac{\sum \text{plaques x dilution factor}}{\sum \text{applied volume}}$$

For example, if the 10^{-4} dilution resulted in 4, 6, 5 and 5plaques foci per well, and inoculated amount was 100 µl, then the stock concentration is

$$\mathbf{((4+6+5+5)/4) \times 10^{4} \times 10 = 0.5 \times 10^{6}\ PFU/ml.}$$

Table 9: Viral titer for RVS infected of HeLa cells

Number of isolate	Viral titer (Pfu/ml)
1	0.8×10^{5}
2	0.6×10^{5}
3	1.1×10^{5}
4	1.7×10^{4}
5	0.6×10^{4}
6	1.0×10^{5}
7	1.8×10^{4}
8	1.1×10^{4}
9	0.6×10^{5}

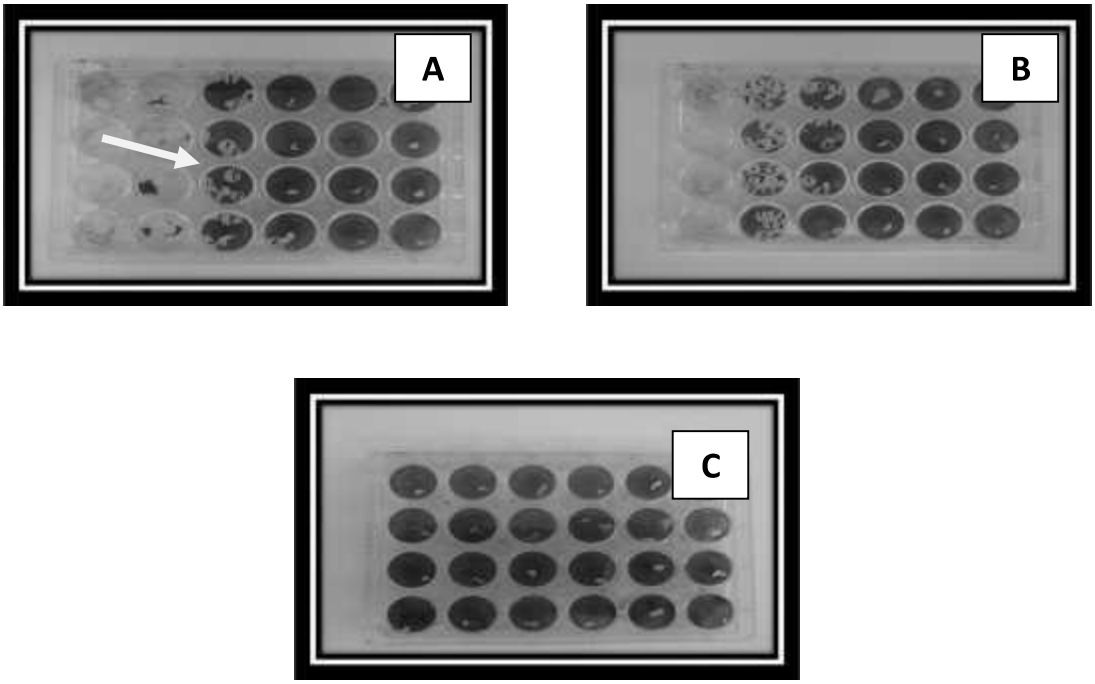


Figure 4: RSV plaques in HeLa cells A. and B. at 6 days post – infection while C. uninfected HeLa cells without plaques (control negative).

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الكشف عن فيروس التنفس المخلوي البشري لدى مرضى الربو باستخدام طريقة الزرع النسيجي وتقنية فحص الوقت الحقيقي – لتفاعل سلسلة البلمرة*.

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علاء هاني الجراخ[1] غانم عبود المولى[2] جلال عبد الرزاق تفاح العزاوي[1]
[1] فرع الاحياء المجهرية, كلية الطب, جامعة بابل, محافظة بابل, العراق
[2] قسم علوم الحياة, كلية العلوم للبنات, جامعة بابل, محافظة بابل, العراق

الخلاصة: هدفت الدراسة الحالية الى التحري عن دور وحدوث فيروسي الجهاز التنفسي المخلوي لدى مرضى الربو المتفاقم في محافظة واسط ، العراق. تم اختبار عينات الدم المحيطي لكل من مجموعتي المرضى والأصحاء لقياس عدد كريات الدم البيضاء الكلي والتفريقي، إذ أظهرت النتائج وجود فروق معنوية في قابلية الخلايا البيضاء بين مجموعة المرضى ومجموعة الأصحاء. فضلا عن ذلك ظهرت فروق معنوية في كل من خلايا الدم البيضاء وهي العدلات واللمفاويات والحمضات والخلايا القاعدية بين مجموعتي الدراسة المختلفة.باستخدام فحص الامتزاز المناعي المرتبط بالإنزيم (الاليزا) على مصل الدم لفحص الأجسام المضادة نوع هـ، أظهرت وجود فروق معنوية بين نتائج مجاميع المرضى مقارنتا بمجاميع الأصحاء. جمعت 80 مسحة مرضية من الأنف والحنجرة ووضعت على الفور في الوسط الناقل الخاص بالفيروسات وخزنها لحين فحصها بفحص تفاعل سلسلة البلمرة بالوقت الحقيقي وجد ان نسبة الإصابة بفيروس كانت 15 مريضا (18.75%). اما طريقة عزل فيروس بطريقة الزرع خط الخلية هيلا أحادي الطبقة، خلايا معزولة من سرطان عنق الرحم البشري، اذ ظهرت تأثير الاعتلال الخلوي الواضح في طبقة الخلايا. وكانت النتائج هي عزل الفيروس 9 عينات (11.25%). باستخدام فحص تفاعل سلسلة البلمرة بالوقت الحقيقي مرة أخرى وجد ان جميع العينات المعزولة بطريق زرع الأنسجة أعطت نتيجة ايجابية إلى الفيروس. الامر الذي يبين وجود الاصابة بالفيروس وعلاقته بمرضى الربو.

كلمات مفتاحية: فايروس التنفس المخلوي البسري, ربو, IgE ,الزراعة على الخط الخلوي نوع هلة.