

# Isolation and identification of Respiratory syncytial virus from Infants with histopathological studies of the isolated virus on experimental animals

Shony M. Odisho<sup>1</sup>PhD , Anton S. Al-Bana<sup>1</sup> PhD, Nahi Y. Yaassen<sup>2</sup> PhD.

## Abstract

**Background:** Human Respiratory syncytial virus (HRSV) is one of the major causes of severe bronchiolitis and pneumonia in infants.

**Objective:** Isolation of virus from specimens from infants with severe bronchiolitis and pneumonia, and study the histopathological changes in laboratory animals.

**Methods:** Specimens collated from infants with lower respiratory tract infection were tested with Respi-Strip kit for the presence RSV antigens; the positive samples were inoculated in HEP-2 for 6-7 passages, and using neutralization test and fluorescent antibody techniques for detection of the isolated virus.

Also 20 mice's 10 weeks old divided into 2 groups one inoculated by dropped in nasal 0.5 ml of 100 TCID<sub>50</sub>/ml and the other 0.5 ml media as control. The lungs removed for histopathology study and the isolation of the virus in 2-7 days after inoculation.

**Results:** The Human RSV was successfully isolated in HEP-2 cell line from five specimens collected from infants with respiratory tract infection, previously tested for presence of RSV

antigen by using Respi-Strip kits, where viral cytopathic effect (CPE) was first detected on 3rd passage with characteristic giant cell or syncytia type formation after 3 days post inoculation. Viral isolates were identified by using homologous reference antiserum by applying indirect immunofluorescent technique (IFAT) and neutralization test (NT).

Experimental infection of mice with the isolated virus revealed histological changes in infected lung mainly characterized with evidence of acute interstitial pneumonia; in addition viruses were re-isolated from infected lung specimens after 2-7 days of experimental infection.

**Conclusion:** Human RSV replicated well in HEP-2 and cytopathic effect appeared in passage three, also the virus can be isolated and cause pathologic change in lung of infected mice.

**Key words:** RSV, HEP-2, Neutralization test.

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## Introduction

Human Respiratory Syncytial Virus (HRSV) is a member of the Pneumovirus subfamily Paramyxoviridae. It account for approximately 50% of all pneumonia and up to 90% of the reported cases of bronchiolitis in infancy<sup>(1)</sup>.

<sup>1</sup>Dept. Microbiology, college of veterinary medicine, Baghdad University, <sup>2</sup>Iraqi centers for cancer and medical genetic researches.

Address Correspondence to: Dr. Shony M. Odisho

E- mail: [shony\\_odesho@hotmail.com](mailto:shony_odesho@hotmail.com)

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Outbreaks of RSV disease are abrupt in onset and can last up to 5 months; the RSV is mainly associated with bronchiolitis in children suffering from underlying illnesses such as congenital heart disease and broncho-pulmonary dysplasia which are at increased risk for severe infection<sup>(2)</sup>. The HRSV is also an important cause of community acquired pneumonia among hospitalized adults of all age<sup>(3)</sup>. The virus was recovered first from children in Baltimore and suggested the name respiratory syncytial virus (RSV) to reflect the giant syncytia which formed in tissue culture<sup>(4)</sup>.

In our country we noticed high percentage of anti RSV and viral antigen in infants under 2 years suffering from lower respiratory infections and bronchiolitis with peak in winter and extends into the spring, and with increasing suspicious of this virus in causing respiratory infections in human; we study the isolation of HRSV from clinical samples of respiratory tract infection from infants in cell culture, study their specific cytopathogenicity, and their histopathological changes in experimentally infected mice.

#### **Materials and methods**

A cross-sectional study was conducted by taking nasal and throat swabs from (100) children with respiratory illness during month (December-March) in 2005-2006, from central pediatric Hospital in Baghdad, the swabs were immersed into tube containing 2 ml cooled transport media with fetal calf sera, and examined with Respi-Strip test: this kit used for detection of human RSV in nasopharyngeal specimens. Five strong positive samples for HRSV were clarified by centrifugation at 3000 xg for 30 minutes at 4 °C, treated for 30 minutes at 37 °C with 500 I.U. Penicillin, 500ug, Streptomycin/ml and centrifuged again at 4 °C for 30 minutes at the same speed, the supernatant was used to inoculated into susceptible cells. HEP-2 cell line: were grown in growth medium RPMI with HEPES, supplemented with 10% fetal calf serum, 100 I.U. penicillin and 100 ug streptomycine/ml. The cells were grown in 25 cm<sup>2</sup> falcon flasks, after complete monolayer cells then were used for detection virus growth, sample of HEP-2 cells were also grown on cover slips in Leighton tubes for detection of viral antigen by indirect fluorescent antibody technique.

The 5 samples were inoculated into HEP-2 cell line as 0.5ml / flask. Control cell cultures were treated with 0.5 ml / flask of maintenance media. Inoculated flasks were incubated at 37 °C for 1-2 hour for virus adsorption (with continuous rolling every 10 minutes) the cells then washed three times with maintenance media. Inoculated and 2 control cultures were fed with 10ml of maintenance medium and incubated at 34 °C and were checked daily for virus growth for 5-7 days by detection of cytopathic effect (CPE).

At the end of incubation time, infected culture was frozen and thawed and 0.5ml of medium, cells, and cell debris were used to repassage into new cell culture flask and treated in the same method of first infection at each passage level. This procedure was repeated three times for the secondary HEP-2 until CPE observed. If CPE were detected hemadsorption test was followed.

Leighton tubes were seeded with HEP-2. After complete monolayer cell growth was reached, the medium was discarded and each tube was inoculated with 0.2ml of the isolated virus suspension and after 1 hour incubation at 37 °C, maintenance medium was added and incubated at 34 °C for 24-72 hours.

Heamdsorption test was done. The medium was removed from the monolayer cell culture flasks and the isolated virus suspension was used to infect these cell cultures. After 24 hour of infections, the monolayer cells were washed three times with warm PBS. Guinea pig erythrocytes were washed three times and prepared as 1% suspension; 10ml of these erythrocytes were added to each flask of the monolayer cells and incubated for one hour at room temperature. The supernatant fluid was removed and

flasks were washed twice with PBS then examined by inverted microscope for evidence of adsorption of RBCs to the infected monolayer cells <sup>(5)</sup>.

When the CPE was observed in cell culture after inoculation by isolated viruses, the cells were fixed with buffered 10% formalin and stained with 1% crystal violet for 24 hour. Giemsa stain was also used after fixation of infected monolayer with methanol for 5-10 minutes then added Giemsa 1% was used to stain cells for 24 hours then washed with tap water and examined by inverted microscope.

Florescent test used for identification of isolated viruses. Discarding the medium from Leighton tubes and then washing the cover slips three times with PBS and the cells fixed with cold acetone for 10 minutes at 4 °C.

Drying of the cover slips in air and treatment with Reference anti-RSV immune serum, incubation for 1 hour at 37 °C in humid chamber. The cover slips washed three times with PBS for 30 minutes and air dried. Goat Anti- human IgG FITC conjugate was added to the cover slips, incubated for 1 hour at 37 °C in a humid chamber. Cover slips washed three times with PBS for 30 minutes and air dried. Dried cover slips were mounted on slides by using 50% glycerin in PBS. The slides were examined by fluorescent U.V. light microscope (Olympus).

Micro titration method was used for titration of isolated viruses; flat bottom 96 wells micro-titer plates were used <sup>(6)</sup>. Cultures of HEP-2 cell line were treated with Trypsin-Versin solution. Sufficient amount of growth medium (about 25 ml for 75 cm<sup>2</sup> flask) was added for each flask. After complete cell dispersion, 0.1ml amount of cell suspension was added for well. When monolayer cell

culture complete, growth media was discarded and the virus suspension was serially 10 fold diluted, inoculated into 4 wells with each dilution (50ul per well) Control cell wells were also used which inoculated with media instead of virus. The plates were covered with sterile adhesive cover and incubated at 37 °C for 1 hour for virus adsorption and then 0.1 ml of maintenance medium added and the plate were covered again and incubated at 34 °C. Virus titer was calculated <sup>(7)</sup>, and the titer was expressed as the highest dilution of virus suspension which showed 50% CPE in infected cell cultures.

Reference antiserum for HRSV Imported from DIALAB Company (Germany) was tested against the locally isolated virus. Neutralization test was carried out by using two fold dilution of reference antiserum against 100 TCID<sub>50</sub> of the isolated virus. The mixtures (0.5ml of each) were incubated at 37 °C for 1 hour before inoculation into 4 wells cell cultures per serum dilution. The antibody titer was expressed as the reciprocal of the highest serum dilution which showed neutralization in 50% of infected cell culture wells.

Healthy (20) ten weeks old mice were supplied from the cancer research institute were divided into 2 groups, first group include 15 mice were inoculated intranasal with 10<sup>5</sup>TCID<sub>50</sub>/ 50ul of HRSV, and the second group include 5 mice inoculated normal cell suspension as control. The animals were examined daily and were killed 1-7 days after inoculation. The Lungs were removed and one lobes used for virus isolation which was homogenized with maintenance media and inoculated into susceptible cells. The other lobes will be fixed in buffered 10% formalin for 24 hour for histopathological examination.

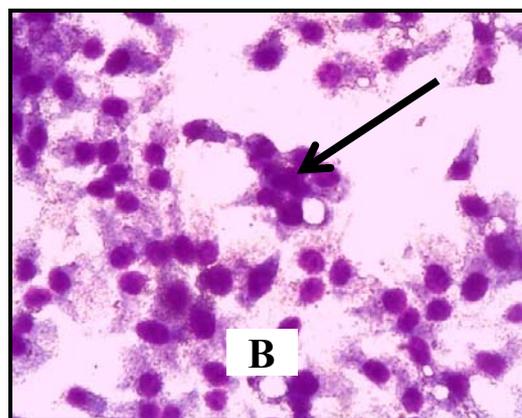
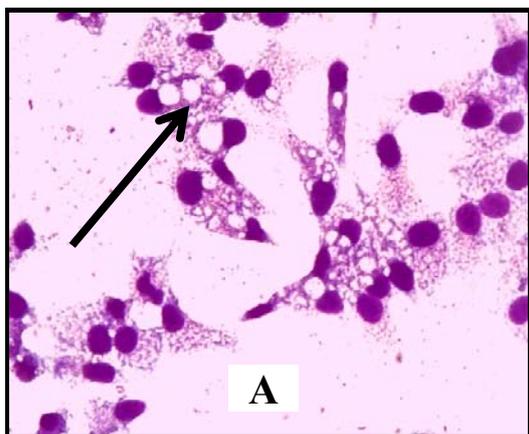
The tissue was then embedded in low- melting point paraffin, sectioned at 5  $\mu$ m thickness, and stained with Hematoxylin and Eosin.

**Results**

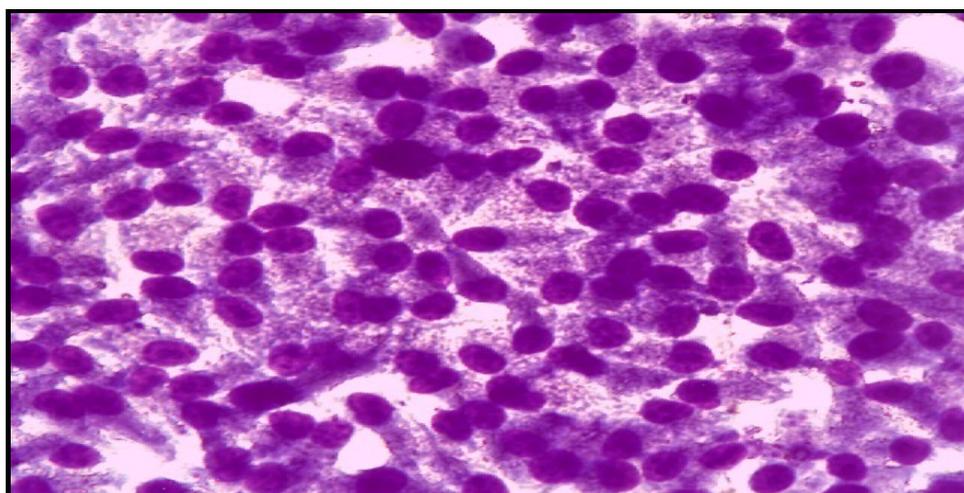
HEP-2 cell line was used for isolation of HRSV from the collected five nasal/ throat swabs taken from infants with acute respiratory tract infection which showed previously strong positive results for viral antigen by RSV-Respi test. In first passage all samples showed no cytopathic effect (CPE). But few rounded cell were noticed in second passage, however clear CPE was first noticed on the 3<sup>rd</sup> passage

after three days, which characterized by cell granulation, aggregation, and separation of the infected cells in culture media.

The number of floating and syncytia cells increased in number on 5th and 6 th passage of HRSV isolates in HEP-2, which can be detected in 48h P. I. and syncytia cells increase in size with subsequent passages, accompanied by formation of empty open plaques which fuses to form large empty spaces with excessive floating cells, but such changes were not detected on control cell culture (Figures 1 and 2)



**Figure 1: Cytopathic effects in HEp-2 cell line infected with the isolated HRSV, 5 days P.I.(A) Vacuolation of cytoplasm (→).(B) Syncytia or giant cells formation (→).(H&E) (x200)**



**Figure 2: Uninfected HEp-2 cell line (H&E) (x200).**

Monolayer cell cultures of HEP-2 infected with the isolated HRSV (passage 5) were used for haemadsorption test. The isolated viruses' infected HEP-2 cells were not able to adsorb Guinea pig erythrocytes after washing of the infected cells.

The titer of isolated HRSV in infected HEP-2 cell line was  $2 \times 10^{2.4}$  TCID<sub>50</sub>/ml in the 3rd passage; Virus titer increased with further passage and reached the maximum titer  $2 \times 10^{5.4}$  TCID<sub>50</sub>/ml at 6th passage (Table 1).

**Table 1: Propagation and titration of HRSV in HEP-2 cell line.**

Passage number	Virus titer TCID <sub>50</sub> /ml
2	$2 \times 10^{1.5}$
3	$2 \times 10^{2.4}$
4	$2 \times 10^{3.2}$
5	$2 \times 10^{5.4}$
6	$2 \times 10^{5.4}$

The demonstration of specific viral antigen of HRSV in infected cell culture at each passage was accomplished by indirect fluorescent antibody technique (IFAT) of infected HEP-2 cell culture grown on cover slips; few fluorescent cells were detected in second and third passage, but 80% of the monolayer cells. In 5th passage of the infected cells showed bright cytoplasmic fluorescence with predominance around the nucleus and occupied most of the cytoplasm.

Reference HRSV antiserum neutralized the infectivity of the isolated HRSV virus in HEP-2 cell culture by using 100 TCID<sub>50</sub>/0.05 with neutralizing titer of 256.

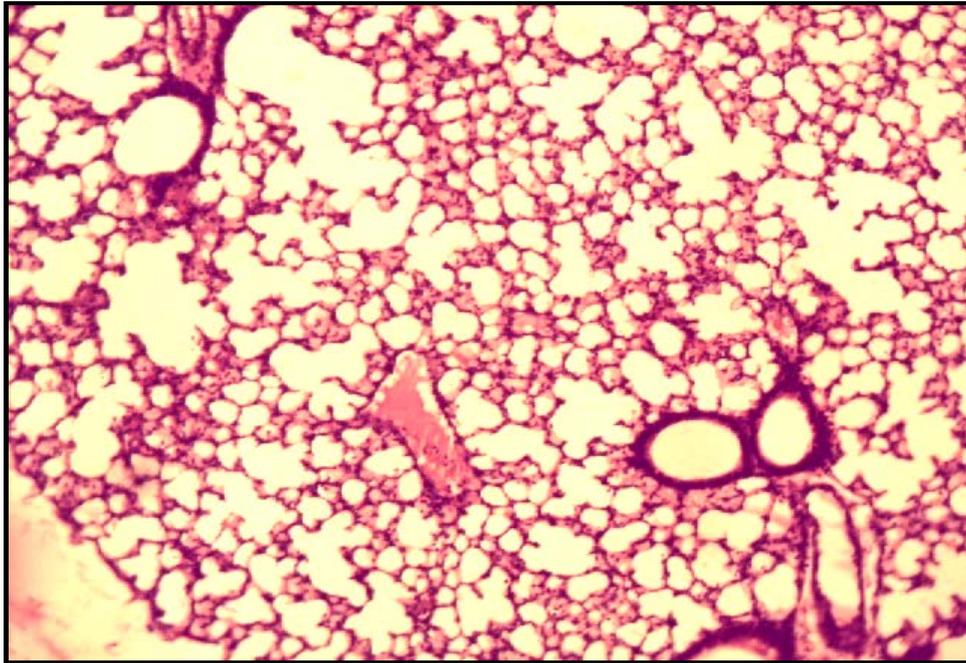
Human RSV was re isolated in HEP-2 from lung samples collected from experimentally infected mice started from 2nd day post inoculation to 7th day.

Histopathological finding in sections of infected lung collected on 2-7 days P.I.

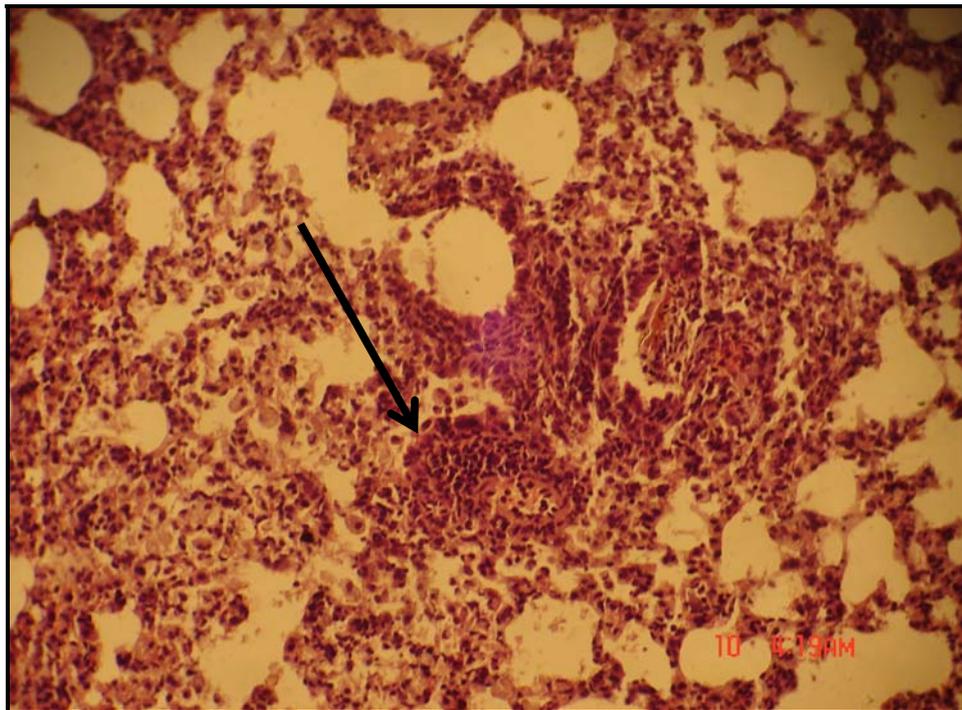
showed evidence of acute interstitial pneumonia which was observed in HRSV- infected lung.

The lesions consisted of an extensive pulmonary edema and congestion accompanied with emphysema. In addition to perivascular leukocytes cuffing, (Figure 4).

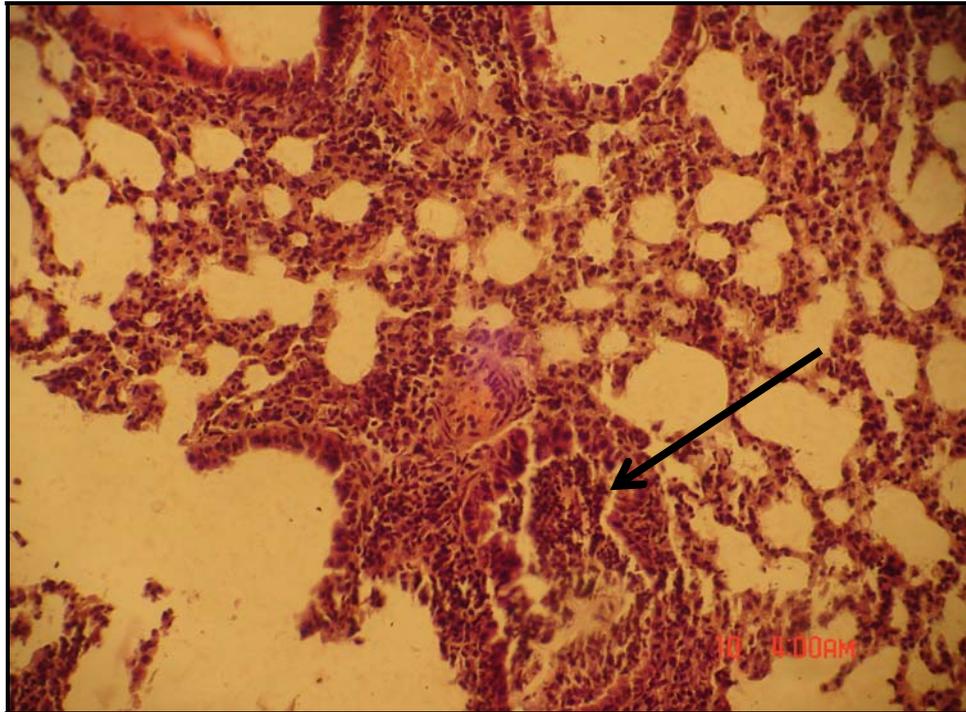
In certain section there was acute bronchiolitis characterized by an infiltration of the neutrophils mainly through bronchiolar wall and in the lumen in addition to sloughing of their epithelial lining, (Figure 5). Both of these lesions were developed into acute interstitial pneumonia, which characterized by thickening of alveolar walls due to infiltration of neutrophils and some lymphocytes and extensive congestion of alveolar capillaries leading to narrowing of alveolar lumena, (Figure 6). These pathological finding were not seen in sections of normal mice lung (Figure 3).



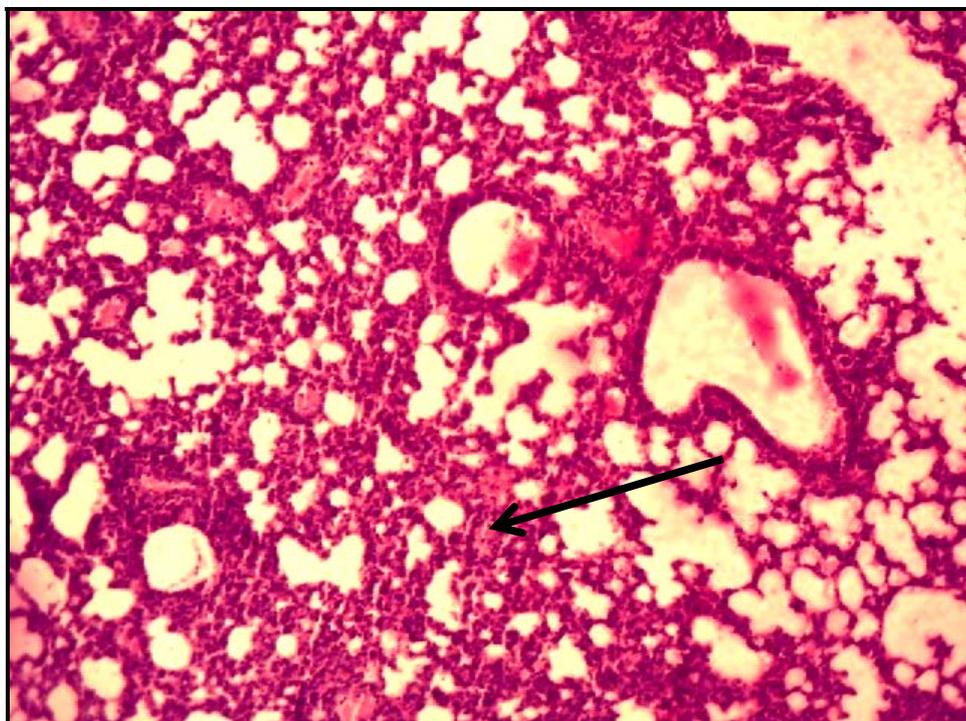
**Figure 3: Section of normal mice lung. (H&E) (x100).**



**Figure 4: Section of infected lung, pulmonary edema with perivascular leukocytes cuffing (→). (H&E)(x100).**



**Figure 5: Section of infected lung with acute bronchiolitis (→) characterized by infiltration of neutrophils through bronchiolar wall and lumen. (H&E)(x100).**



**Figure 6: Section of infected lung with acute interstitial pneumonia (→), characterized by thickening of alveolar walls due to infiltration of neutrophils and lymphocytes. (H&E)(x100).**

### **Discussion**

The HRSV was isolated from 5 positive specimens' in HEP-2 cell line culture as these cells found to be sensitive for viral isolation. Our results agreed with other studies which proved that HEP-2 was supporting growth of HRSV and the CPE appeared after sub passages and after 5 days PI<sup>(8,9)</sup>.

As reported RSV infected HEP-2 cells needs 34°C for incubation to support replication of our viral isolates of HRSV, the virus titer gradually increased with sub passage and reach its maximum titer 10<sup>(5,4)</sup> TCID<sub>50</sub>/ml, our result agree with other studies in increased of titer in subpassage of virus in HEP-2 at 34°C but differ from others who obtained high titer, this difference in virus titer could be due to variation in viral strains or viral subgroup which studies show that group A replicate better than group B in cell culture<sup>(8,10)</sup>. Also many factors affect rate of virus isolation as timing factor in collecting of the samples, due to loss of viral infectivity because of viral liability which was avoided by immediate inoculation of specimens into susceptible HEP-2 cells, it was found that 90% reduction in the virus titer within 2 hours was observed, but 90% reduction occurred within 24-48 with the virus suspended in medium 199 with 5% rabbit serum<sup>(8,9)</sup>. Our result of HRSV isolation is in agreement with others and was successful by using MEM medium supplement with 5% fetal calf serum as transport media<sup>(9,10)</sup>, also using nasal/throat swabs for virus isolation was very beneficial in getting positive results in virus isolation which agreed with positive detection of viral antigen by Respi-test. However studies have reported that nasopharyngeal wash was proved to carry 500 fold higher in virus

content than nasal swabs specimens<sup>(10,11)</sup>.

The HRSV, infected cell culture had showed no activity of the viral isolates to haemadsorb guinea pig erythrocytes. This is related to the probable characters of HRSV in absence of haemagglutinin which differ from other paramyxoviruses and also to exclude the presence of such viral infection<sup>(12)</sup>.

Identification of the isolated viruses was performed by indirect immunofluorescent technique which appeared as bright cytoplasmic fluorescence in susceptible infected cells mainly around the nucleus in HRSV, Also virus neutralization test was used for identification the isolated HRSV as indicated in our result that reference antiserum for HRSV neutralized the infectivity of viral isolates<sup>(13)</sup>.

Human RSV was reisolated in HEP-2 from lung samples collected from experimentally infected mice started from 2-7 days PI. These result proved that mice (8-10 weeks) can be experimentally infected with human RSV local isolates as it was indicated by reisolation of virus from target organ with the classical histopathological changes, these agreed with other studies used mice and cotton rats as experimental animals in their investigations<sup>(14,15)</sup>. These findings agree with several studies which discussed the pathogenicity of HRSV inoculation of various strains of inbred mice, all described the moderate influx of inflammatory cells with reports including lymphocytes and eosinophiles as components of respiratory infiltration<sup>(16,17)</sup>.

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