AIM OF THE WORK

The aim of the present work is the molecular diagnosis of Human Metapneumovirus (hMPV) infection using a real-time, reverse-transcription PCR assay, and the evaluation of it's role among Egyptian infants (age $\leq$ 2 years) clinically diagnosed with acute viral bronchiolitis. Clinical impact of coinfection with other common respiratory viruses will be also studied using a qualitative indirect immunofluorescence screening assay.

PATIENTS AND METHODS

Study Design:

This study, carried out during the period from January to March 2013. It included fifty Egyptian infant's attending the Alexandria University Children's Hospital of El-Shatby (Alexandria, Egypt), and clinically diagnosed with acute viral bronchiolitis during the winter/spring season of this year.

• Patients and Data Collection:

The study included infants who were clinically diagnosed with acute viral bronchiolitis according to the following criteria:

1. Age less than two years.
2. First attack of wheezy chest.
3. Absence of response to bronchodilators.

Prematurely born or immunocompromised subjects, and/or subjects with chronic pulmonary or congenital heart diseases were all excluded from the study. Subjects whose parents/guardians refused to enroll their children in the study were also excluded. The data collected for each child included personal data (age, sex... etc) as well as a full past medical history. Each child was then subjected to a clinical examination, followed by appropriate laboratory and radiological examination. A nasopharyngeal aspirate and a throat swab were then collected from each child.

• Ethical Considerations:

Under the guidelines of the "Committee of Ethics" of Faculty of Medicine, Alexandria University, an informed consent from the parents of each child was obtained prior to sample collection and after a brief explanation of research objectives and study purpose.

Laboratory Methods:

From each child, two samples were collected; one was a nasopharyngeal aspirate that was collected using a sterile mucus extractor and screened immediately through a qualitative indirect immunofluorescence assay (IMAGEN^{TM} Respiratory Screen Kit)[93] for the purpose of detecting common respiratory viruses, other than hMPV, that might be involved in infection. The second one was a throat swab collected using the standard plastic-shafted virocult® swabs, separated into aliquots and kept frozen at -70^{o}C for further PCR analysis. A real-time, reverse-transcription PCR assay (Primer Design^{TM} genesig qPCR Detection Kit) was used to detect the hMPV nucleic acid in patient's respiratory specimens.[94]

Antigen detection assays:

The Immunofluorescence Respiratory Screen Test

The IMAGEN™ Respiratory Screen (Oxoid, Hampshire, UK) is a qualitative indirectimmunofluorescence test for the presumptive detection of RSV, influenza A and B virus, para-influenza virus 1, 2 and 3 and adenovirus in respiratory specimens (nasopharyngeal aspirates) and in cell cultures. The assay cannot differentiate between the viruses. Individual viruses can then be identified and confirmed using monospecific FITC labelled monoclonal antibodies.

- **Test Principle:**

The IMAGEN™ Respiratory Screen test contains a pool of monoclonal antibodies each of which has individual specificity for either RSV, influenza A or B virus, parainfluenza virus 1, 2 or 3 or adenovirus. The pooled antibody Screening Reagent is used in a two-step indirect immunofluorescence staining technique [95] (Fig 8).

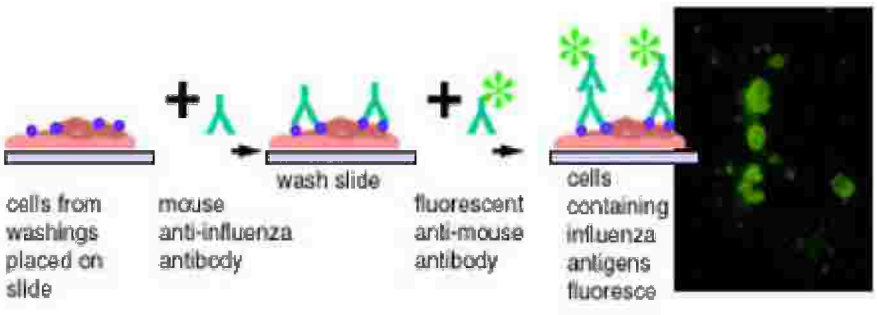


Figure (8): Schematic Diagram of Indirect Immunofluorescence Assay

The Negative Control Reagent provided is used to monitor the specificity of staining. Specimens are stained with the Screening Reagent and/or Negative Control Reagent for15 minutes. Excess unbound reagent is then removed by washing with phosphate buffered saline (PBS). The specimens are then stained with a secondary FITC conjugate for 15 minutes. Excess unbound reagent is removed by washing with PBS. The stained areas are mounted and viewed microscopically using epifluorescence illumination. A positive result is indicated by the presence of characteristic apple-green intracellular fluorescence within infected cells which contrasts with the red background staining of uninfected cells. The respiratory virus present can then be specifically identified using individual IMAGEN™ reagents.

- **Reagents:**

A) IMAGEN™ Respiratory Screen Kit:

Each kit contains sufficient materials for 100 direct specimens or cell culture preparations, and consists of the following components:

i. **Positive and Negative Control Slides:** 5x14 wells, combined Positive and Negative Control Slides. Each slide consists of 7 wells containing acetone-fixed cells infected with either RSV (strain from clinical specimen), influenza A (strain CDC V7-002) or B virus (strain CDC V4-004), parainfluenza virus 1, 2 or 3 (strains CDC V6-004,

CDC V7-003, and CDC V5- 003 respectively) and adenovirus (strain CDC V5-002), one well specific for each virus) and 7 wells containing acetone fixed uninfected cells (negative control wells).

ii. **Mounting Fluid:** A3x3mL of mounting fluid containing a photobleaching inhibitor in glycerol (pH 10.0).

iii. **Screening Reagent:** A4.4mL of screening reagent consisting of a pool of purified mouse monoclonal antibodies specific for RSV, influenza A and B virus, parainfluenza virus 1, 2 and 3 and adenovirus. The reagent is prepared in a protein stabilized buffer solution containing 15mmol/L sodium azide as a preservative.

iv. **Negative Control Reagent:** A2.6mL of negative control reagent consisting of pooled mouse monoclonal antibodies with no anti-viral activity. The reagent is prepared in a protein stabilized buffer solution containing 15mmol/L sodium azide as a preservative.

v. **FITC Conjugate:** A 2x3.5mL of anti-mouse FITC conjugated antibody reagent consisting of an FITC conjugated F(ab')2 fragment of rabbit antimouse immunoglobulins, diluted in PBS solution containing stabilizing proteins, Evans blue dye as a counter-stain and 15mmol/L sodium azide as a preservative.

B) Additional Reagents:

1. Fresh acetone (for fixation).
2. Fresh phosphate buffered saline (PBS) pH 7.5 prepared on the day of use for washing stained specimens and for specimen preparation.

- **Equipment Used:**

The following equipment was used in the assay:

1. Mucus extractor used to obtain the specimens (nasopharyngeal).
2. Precision pipettes and disposable tips used to deliver 20µL and 25µL.
3. Teflon coated glass microscope slides with single 6mm diameter wells.
4. Cover slips to cover either single 6mm diameter wells or 14 well slides.
5. Non-fluorescing immersion oil.
6. Epifluorescence microscope with filter system for FITC (maximum excitation λ 490 nm, mean emission λ520 nm) and x200-x500 magnification.
7. Low speed centrifuge.
8. Incubator at 37°C.
9. Wash bath.

- **Specimen Collection and Slide Preparation:**

Specimens were collected from the nasopharyngeal region into a mucus extractor through a size 8 feeding tube. The mucus extractor and tubing were transported as soon as possible, maintained at 2-8°C and sent to the Immunology department's laboratories at the Alexandria University MRI.

The specimens were immediately processed as shown in the following steps:

1. About 2mL PBS were first added to the specimen prior to centrifugation to reduce the viscosity and dilute the mucus.
2. The mucus extractor was then centrifuged at room temperature (15-30°C) for 10 minutes at 380 x g.
3. The supernatant was removed, and the cell deposit was resuspended in 2mL PBS.
4. The cells were then gently pipetted up and down with a wide bore pipette, or a gentle vortex, until the mucus was broken up and cellular materials were released.(N.B: Vigorous pipetting/vortexing was avoided to prevent damage to the cells).
5. When a smooth suspension has been obtained, further PBS was added as required. Pipetting or vortexing was then done (after addition of the extra PBS) to wash the cells further.
6. Any visible flecks of mucus remaining at this point were then removed and discarded, since any excess mucous will prevent adequate penetration of the reagent and may result in non-specific fluorescence.
7. After completing the cell separation process, the resultant cell suspension was centrifuged at room temperature (15-30°C) for 10 minutes at 380 x g and the supernatant was discarded.
8. The resulting cell deposit was resuspended in sufficient PBS to dilute any remaining mucus, while at the same time maintaining a high cell density.
9. 25µL of the resuspended cell deposit were then placed into the well area on the slide.(N.B: It was important that duplicate wells were prepared for each specimen to be tested using the IMAGEN™ Respiratory Screen, one well for Screening Reagent and one well for Negative Control Reagent).
10. Each specimen was allowed to air dry thoroughly at room temperature (15-30°C) and then fixated in fresh acetone at room temperature (15-30°C) for 10 minutes. In case the specimens were not stained immediately, the slides were stored at 4°C overnight or frozen at -20°C for longer storage periods.

- **Test Procedure:**

Preparatory work step: The kit reagents and control slides were first left at room temperature (15-30°C) for 5 minutes before being used.

- **Step (1) - addition of screeening reagent and negative control reagent:** For each specimen two wells of fixed cell preparation were required. To one well of the test slide, 25µL of the Screening Reagent was added, and 25µL of the Negative Control Reagent was added to the other well. To the control slide, 20µL of the Screening Reagent was added to each well.
- **Step (2) - first incubation:** The slides were incubated with reagent in a moist chamber for 15 minutes at 37°C. (N.B: It was important not to allow the reagent to dry on the specimen, as this will cause the appearance of non-specific staining).
- **Step (3) - washing the slide:** Excess reagent was washed off with PBS. The slide was then gently washed in an agitating bath containing PBS for 5 minutes. PBS was then drained off and the slide was allowed to air dry at room temperature (15-30°C).
- **Step (4)- addition of anti-mouse fitc conjugate:** 25µL of the FITC reagent was added to each specimen well and only 20µL of it was added to each well of the control slide

(since control slides have wells with adiameter of less than 6mm and it was recommended that only 20µL of reagent is used).

- **Step (5) - second incubation:** The slides were incubated with FITC reagent in a moist chamber for 15 minutes at 37°C. As in the first incubation, the reagent wasn't allowed to dry on the specimen, as this will cause the appearance of non-specific staining.
- **Step (6) - washing the slide:** The same as step (3).
- **Step (7) - addition of mounting fluid:** One drop of the mounting fluid was added to the centre of each well and a cover-slip was added over the mounting fluid and specimen ensuring that no air bubbles were trapped.
- **Step (8) - reading the slide:** The entire well areas containing the stained specimen were examined using an epifluorescence microscope. As mentioned earlier, fluorescence should be visible at x200-x500 magnifications.

• Interpretation of Results:

For control slides, positive wells should show cells with intracellular apple-green cytoplasmic and/or nuclear fluorescence contrasting against a background of redcounter stained material. Positive control slides should be used to check that the staining procedure has been satisfactorily performed. Negative wells, on the other hand, should show cells with no intracellular apple-green fluorescence. Only background red counterstaining should be visible.

For clinical samples, stained infected cells will demonstrate apple-green cytoplasmic and/or nuclear fluorescence. Uninfected cells stain red with Evans blue counter stain. A positive diagnosis is made when one or more cells in the fixed, stained specimen show this typical fluorescence pattern. A negative diagnosis is made when fixed, stained specimens do not exhibit fluorescence with the screening reagent. For directly stained nasopharyngeal aspirate specimens, at least 20 uninfected respiratory (columnar) epithelial cells must be observed within each slide well area before a negative result is reported. [96]

Molecular detection methods:

Real-Time Reverse-Transcription PCR Assay

A real-time RT-PCR assay remains to be the most sensitive, rapid and reliable diagnostic laboratory test used for detecting hMPV. The Primer Design™ genesig qPCR Detection Kit (Primer Design, Southampton, UK), was used in our present work to molecularly detect hMPV in patients nasopharyngeal specimens by targeting and amplifying the hMPV nucleoprotein (N) gene.[98] All work procedures were performed at the Microbiology department of the Medical Research Institute, Alexandria University. The primer and probe mix provided in Primer-Design™ genesig qPCR Detection Kit exploits the TaqMan® principle. [98]

• Sample Collection and Preparation:

Using the flexible aluminum-shafted Virocult® swabs [98] (Medical Wire and Equipment, Wiltshire, UK), a nasopharyngeal sample was obtained from each patient enrolled in our study. Each Virocult® unit consists of a sterile swab and a prelabelled transport tube which contains a foam pad saturated with 1.2ml of transport medium

(balanced salt solution plus glucose, lactalbumin hydrolysate and antibiotics to suppress bacterial and fungal growth).

For processing the specimens, approximately 2.0ml of Eagle's or Hank's solution were added to the transport tube with the swab in situ. The tube contents were then mixed thoroughly by squeezing the foam pad in the base of the tube several times. Using a pipette the liquid was withdrawn, distributed into separate aliquots (approximately 0.2ml of the suspension in each) and kept frozen at -70 °C for further PCR analysis.

- **RNA Extraction:**

Extraction and purification of hMPV RNA was performed using the QIAamp® Viral RNA Mini-Kit (QIAGEN, Hilden, Germany), according to the QIAamp® Viral RNA Mini Spin Procedure illustrated in the manufacturer's handbook. Before being processed for RNA extraction, samples were first brought at room temperature, centrifuged for 10 minutes at 1500 x g and the supernatant was used.

A. Kit Contents:

1. 50 QIAamp Mini Spin Columns
2. 200 Collection Tubes (2ml)
3. Buffer AVL (31ml)
4. Buffer AW1 (concentrate) (19ml)
5. Buffer AW2 (concentrate) (13ml)
6. Buffer AVE (3x2ml)
7. Carrier RNA (poly A) (310μg)

B. Additional Reagents and Equipment Used:

1. Ethanol (96-100%).
2. 1.5ml microcentrifuge tubes.
3. Sterile, RNase-free pipette tips.
4. Microcentrifuge (with rotor for 1.5ml and 2ml tubes).

C. Principle of QIAamp® Viral RNA Extraction:

The QIAamp® Viral RNA Mini-Kit combines the selective binding properties of a silicagel-based membrane with the speed of microspin or vacuum technology. [99]

1. **Sample Lysis**: The sample is first lysed under highly denaturing conditions provided by Buffer AVL to inactivate RNases and to ensure isolation of intact viral RNA.
2. **Adsorption to QIAamp Membrane**: The sample is loaded onto the QIAamp Mini spin column, and viral RNA is adsorbed onto the QIAamp silica membrane during two brief centrifugation steps or by vacuum. Buffering conditions are adjusted to provide optimum binding of the RNA to the QIAamp membrane. Salt and pH conditions in the lysate ensure that protein and other contaminants, which can inhibit downstream enzymatic reactions, are not retained on the QIAamp membrane.
3. **Removal of Residual Contaminants:** Viral RNA, bound to the QIAamp membrane, is washed free of contaminants during two short centrifugation or vacuum steps. The use of two different wash buffers, AW1 and AW2, significantly improves the purity of

the eluted RNA. Optimized wash conditions ensure complete removal of any residual contaminants without affecting RNA binding.

4. **Elusion with Buffer AVE:** High-quality RNA is lastly eluted in a special RNase-free buffer, ready for direct use or safe storage. The purified RNA is free of protein, nucleases, and other contaminants and inhibitors.

D. Preparation of Reagents:

1. Addition of Carrier RNA to Buffer AVE:

310µl of Buffer AVE was added to the tube containing 310µg lyophilized carrier RNA to obtain a solution of 1µg/µl. The carrier RNA was dissolved thoroughly, divided it into conveniently sized aliquots, and stored at -20°C. Carrier RNA serves two purposes; firstly, it enhances binding of viral nucleic acids to the QIAamp Mini membrane, especially if there are very few target molecules in the sample. Secondly, the addition of large amounts of carrier RNA reduces the chance of viral RNA degradation in the rare event that RNase molecules escape denaturation by the chaotropic salts and detergent in Buffer AVL.

2. Addition of Dissolved Carrier RNA to Buffer AVL:

Dissolved carrier RNA was then added to Buffer AVL in volumes indicated in the kit's handbook. This solution (stable at 2-8°C for up to 48 hours) was prepared fresh. Carrier RNA was added to Buffer AVL in order to improve the binding of viral RNA to the QIAamp membrane especially in the case of low-titer samples, and limit possible degradation of the viral RNA due to any residual RNase activity. Since Buffer AVL-carrier RNA develops a precipitate when stored at 2-8°C, it was important to redissolve it by warming at 80°C (NOT more than 5 min.) before use. Frequent warming and extended incubation of the Buffer AVL-carrier RNA was avoided, as this will cause degradation of the carrier RNA, leading to reduced recovery of viral RNA and eventually false negative RT-PCR results.

3. Addition of Ethanol to Buffers AW1 and AW2:

Both Buffers AW1 and AW2 were supplied as concentrates. Before being used for the first time, an appropriate amount of ethanol (96-100%) was added to both buffers as indicated in the kit's handbook.

E. Mini Spin Protocol Procedure:

The procedure isoptimized for the purification of 140µl samples using a microcentrifuge: Before starting, samples and Buffer AVE were brought to room temperature (15-25 °C). [99]

1. 560µl of the prepared Buffer AVL containing carrier RNA were first pipetted into a 1.5ml microcentrifuge tube.
2. 140µl of the sample were then added to the Buffer AVL-carrier RNA in the micro-centrifuge tube, and mixed by pulse-vortexing for 15 sec.
3. The tube was incubated at room temperature (15-25°C) for 10 min, and then briefly centrifuged to remove drops from the inside of the lid.

4. 560µl of ethanol (96-100%) were added to the sample, and mixed by pulse-vortexing for 15 sec. After mixing, the tube was briefly centrifuged to remove drops from inside the lid.
5. 630µl of the solution from the previous step were carefully added to the QIAamp Mini column (in a 2ml collection tube) without wetting the rim. The cap was closed, and the tube was centrifuged at 8000 rpm for 1 min. The QIAamp Mini column was then placed into a clean 2ml collection tube and the tube containing the filtrate was discarded.
6. The QIAamp Mini column was carefully opened, and step 5 was repeated.
7. The QIAamp Mini column was then carefully opened and 500µl of Buffer AW1 were added to it. The cap was closed and the tube was centrifuged 8000 rpm for 1 min. The QIAamp Mini column was then placed in a clean 2ml collection tube and the tube containing the filtrate was discarded.
8. Again the QIAamp Mini column was carefully opened and 500µl of Buffer AW2 were added to it. The cap was closed, and the tube was centrifuged at the full speed (14.000 rpm) for 3 min.
9. In case of possible Buffer AW2 carryover, the QIAamp Mini column was added in a new 2ml collection tube (after discarding the old tube containing the filtrate) and centrifuged at full speed for 1 min.
10. Finally, the QIAamp Mini column was added into a clean 1.5ml microcentrifuge tube (after discarding the old collection tube containing the filtrate). The QIAamp Mini column was then carefully opened and 60µl of Buffer AVE were added after being equilibrated to room temperature. The cap was closed and the tube was incubated at room temperature for 1 min, before being centrifuged at 8000 rpm for 1 min.
11. The resulting eluted solution now contained a high-quality RNA in a special RNase-free buffer, ready for direct use or storage for one year at -20°C or -70°C.

• PCR Instrument:

The PCR instrument model used in our work was the Applied BiosystemsStepOne™ Real-Time PCR System [100] (Applied Biosystems, Inc., Foster City, CA, United States) (Fig. 9). This 48-well platform instrument utilizes a long-life LED-based optical system that can record fluorescence from FAM™/SYBR® Green, VIC®/JOE™, and ROX™ dyes. Regardless of the run type, a data collection point or *read* on the StepOne™ instrument consists of three phases: (a) **Excitation** - The instrument illuminates all wells of the reaction plate within the instrument, exciting the fluorophores in each reaction, (2) **Emission** - The instrument optics collect the residual fluorescence emitted from the wells of the reaction plate. The resulting image collected by the device consists only of light that corresponds to the range of emission wavelengths, and (3) **Collection**- The instrument assembles a digital representation of the residual fluorescence collected over a fixed time interval. The StepOne™ software stores the raw fluorescent image for analysis.

Figure (9): Applied Biosystems StepOne™ Real-Time PCR System [100]

- **Precision OneStep™ qRT-PCR MasterMix:**

For molecular detection and quantification of hMPV nucleic acids, there are two primary methods of real-time, reverse-transcription PCR that can be performed. The first one combines the reverse transcription and real-time PCR reaction in a simple closed tube protocol (one-step). The second method involves creating cDNA first by means of a separate reverse transcription reaction and then adding the cDNA to the PCR (two-step).In our work, a one-step real-time RT PCR protocol was used, as this method is rapid and much simpler saving significant bench time and also reducing possible errors and contaminations. In addition, the need for a separate reverse transcription kit is eliminated which reduces costs dramatically. The sensitivity of a one step protocol is also greater than a two step because the entire biological sample is available to the PCR without dilution.

The Precision OneStep™ qRT-PCR MasterMix Kit (PrimerDesignTM Ltd, Southampton, UK) includes all of the required components for a perfect one step real-timePCR analysis in a single reaction mix. It contains a unique thermo stable MMLV (Moloney murine leukemia virus) enzyme which has an optimal operating temperature of 55°C, and achieves reverse transcription of the template RNA in less than 10 minutes. It also contains *Taq* Polymerase as well as buffer and MgCl2 at concentrations optimized for the amplification step. Only the template RNA and primer and probe mix are required to complete the experimental set up for a perfect single tube reaction. Each kit contains a Precision OneStepTM qRT-PCR Master-mix (which includes thermo-stable MMLV and a complete qPCR mastermix), and a PrecisionTM qPCR Mastermix (which is a control reagent that lacks the MMLV and is used to make an RT minus control). [101]

- **hMPV PrimerDesignTM genesig qPCR Detection Kit:**

The Advanced PrimerDesign™ genesig qPCR Detection Kit (150 reaction tests) for the hMPV Genomes is designed for the in vitro quantification of hMPV genomes. The primers have 100% homology with all reference sequences in the NCBI database and therefore have the broadest detection profile possible whilst remaining specific to the hMPV genome.

A. Kit Contents:

1. hMPV specific primer/probe mix - FAM labeled, BHQ quenched.
2. hMPV positive control template (for Standard curve).
3. Internal extraction control RNA.
4. Internal extraction control primer/probe mix - Choice of Yakima Yellow (VIC dye channel) or Cy5 label.
5. Endogenous ACTB primer/probe mix - FAM labeled, BHQ quenched.
6. Internal extraction control/hMPV/ACTB RT primer mix for 2-step protocol only.
7. RNAse/DNAse free water.

B. Reagents and Equipment Used:

1. Real-Time PCR Instrument.
2. QIAamp® Viral RNA Extraction Mini-Kit.
3. Precision OneStepTM qRT-PCR MasterMix kit.
4. Pipettors and Tips.
5. Vortex and centrifuge.
6. Thin walled 1.5ml PCR reaction tubes.

C. Internal RNA Extraction Control:

When performing RNA extraction, it is often advantageous to have an exogenous source of RNA template that is spiked into the lysis buffer. This control RNA is then co-purified with the sample RNA and can be detected as a positive control for the extraction process. Successful co-purification and real-time PCR for the control RNA also indicates that PCR inhibitors are not present at a high concentration. A separate RT primer mix and a real-time PCR primer/probe mix are supplied with this kit to detect the exogenous RNA using real-time PCR. The PCR primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control cDNA does not interfere with detection of the pathogen target cDNA even when present at low copy number. The Internal control is detected through the VIC channel and gives a Ct value of 31+/-3 depending on the level of sample dilution. In our work, 4µl of the internal extraction control RNA was added either to the RNA lysis/extraction buffer or to the RNA sample once it has been resuspended in lysis buffer. This was followed by a complete RNA extraction protocol, according to the manufacturer's instructions. (N.B: Addition of the internal extraction control RNA directly to the unprocessed biological sample was avoided, as this would lead to degradation and a loss in signal).

D. Reconstitution Protocol:

Each tube was first pulse-spinned in a centrifuge before being opened to ensure that lyo-philised primer and probe mix is in the base of the tube and wouldn't be spilt upon opening the tube. The kit components were then reconstituted as following:

Component:	**Volume**
hMPV Primer/Probe mix	165µl
Internal extraction control primer/probe mix	165µl
Internal extraction control/Pathogen/ACTB RT primer mix	165µl
Endogenous ACTB Primer/Probe mix	165µl

Internal extraction control RNA	600 µl
Positive Control Template	500 µl

To ensure complete resuspension, each tube was vortexed thoroughly.

E. Standard Curve Protocol:

I. For each standard curve sample, a reaction mix was prepared as following:

Component:	**Volume**
2X Precision OneStepTM qRT-PCR MasterMix	10µl
hMPV Primer/Probe mix	1µl
RNAse/DNAse free water	4µl
Final Volume:	**15µl**

II. A standard curve dilution series was then prepared as following:

1. 900µl of RNAse/DNAse free water were pipetted into 5 tubes labelled from 2 to 6.
2. 100µl of the Positive Control Template were pipetted into tube 2 and vortexed.
3. Pipette tip was changed and 100µl from tube 2 were pipetted into tube 3 and vortexed.
4. Steps 2 and 3 were repeated to complete the dilution series as shown below:

Standard Curve:	**Copy Number:**
Tube 1 (POSITIVE CONTROL)	2 X 105 per µl
Tube 2	2 X 104 per µl
Tube 3	2 X 103 per µl
Tube 4	2 X 102 per µl
Tube 5	20 per µl
Tube 6	2 per µl

III. 5µl of standard template were finally pipetted into each well for the standard curve so that the final volume in each well is 20µl.

F. One-step RT PCR Protocol:

This approach combines the reverse transcription and amplification in a single closed tube. For each RNA sample, a reaction mix was prepared as following:

Component:	**Volume:**
2X Precision OneStepTMqRT-PCR MasterMix*	10µl
hMPV Primer/Probe mix	1µl
Internal extraction control primer/probe mix	1µl
RNA sample	Xµl
RNAse/DNAse free water	Xµl
Final Volume:	**20µl**

- To perform an RT negative control, substitute the 2 x Precision OneStepTM qRT-PCR MasterMix for the standard Precision MasterMix (which lacks the RT enzyme).

For each RNA sample, an endogenous ACTB control reaction was prepared as following:

Component:	**Volume**
2X Precision OneStep™ qRT-PCR MasterMix	10µl
Endogenous ACTB* Primer/Probe mix	1µl
RNA sample	Xµl
RNAse/DNAse free water	Xµl
Final Volume:	**20µl**

- The endogenous ACTB control is used to detect the Actin Beta (ACTB) gene, in order to provide crucial information regarding the quality of the biological sample. Detection of ACTB is through the FAM channel (that's why it is not possible to perform a multiplex for ACTB and the pathogen primers). A poor ACTB signal may indicate that the sample did not contain sufficient biological material.

G. One-step Amplification Protocol:

Amplification conditions using the PrimerDesign 2X Precision OneStepTM MasterMix are shown in the following table (Table 1):

Table (1): Amplification Conditions of PrimerDesign 2X Precision OneStep™ MasterMix

	Step	Time	Temperature
	Reverse Transcription	10 min.	55°C
	Enzyme Activation	8 min.	95°C
x50 Cycles	Denaturation	10 sec.	95°C
	Data Collection*	60 sec.	60°C

*Fluorogenic data should be collected during this step through the FAM and VIC channels.

■ Thermal Profile:

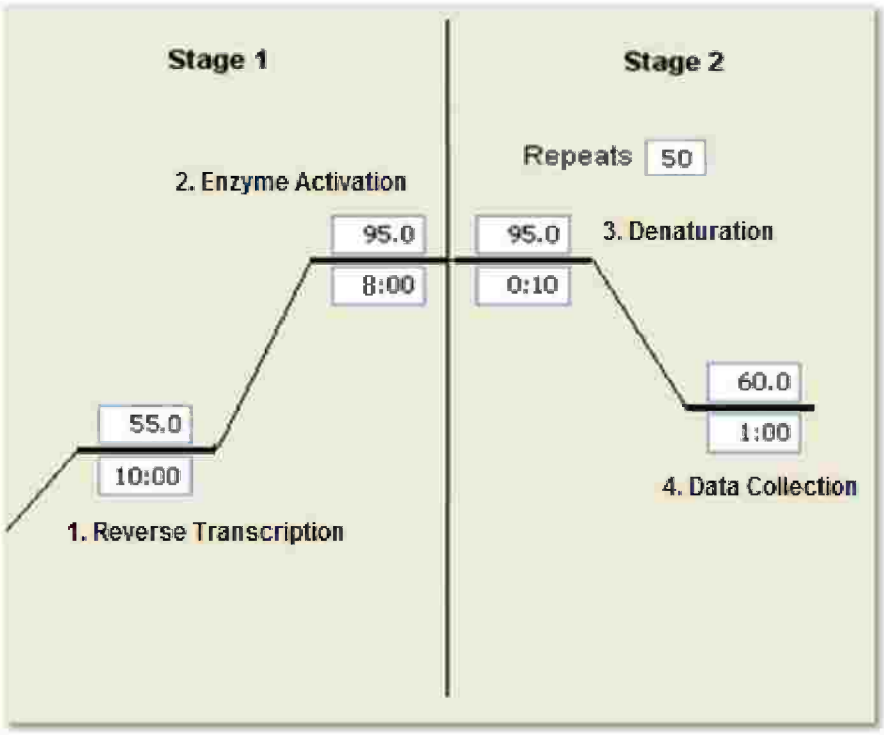


Figure (10): Thermal Cycling Profile of hMPV PCR Assay

Figure (10) shows the thermal cycling profile of the hMPV PCR assay. Stage I of the assay starts with reverse transcription step at 55°C for 10 min, followed by enzyme activation step at 95°C for 8 min. Stage II then begins with the denaturation step at 95°C for 10 seconds, followed by the data collection step at 60°C for 1 min.

H. Interpretation of Results:

In our assay, PCR results were interpreted according to the following table:

Table (2): Interpretation of hMPV qPCR Results

Target	Internal control	Negative control	Positive control	Interpretation
+ve	+ve	-ve	+ve	Positive result
+ve	-ve*	-ve	+ve	Positive result
-ve	+ve	-ve	+ve	Negative result
-ve	-ve	-ve	-ve	Experiment fail
+ve	+ve	+ve	+ve	Experiment fail

*When amplifying an hMPV sample with a high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.

Routine Lab investigation

CBC with differential WBCs:

A phlebotomist collects the samples (2 ml), drawing the blood into a test tube containing an anticoagulant (EDTA) to stop it from clotting. Showing the values of red blood cells, platelets and white blood cells differential.

Quantitive CRP:

Using AVITEX CRP Ref OD073/OD023/E.

Pulse oximetery:

Using NELLCOR APK-WI-02-P-14 reusable pulse oximetery.

To asses heart rate and saturation of Oxygen in blood to asses severity of distress of infant.

Radiological investigation:

Chest X-ray done to exclude other disease.

Statistical Analysis:

A total sample size of 50 Egyptian infant patients, attending the Alexandria University Children's Hospital at El-Shatby during the winter/spring season of 2013, and clinically diagnosed with acute bronchiolitis, was used in the present study. The resulting data were organized and processed by the Statistical Package for the Social Sciences (SPSS) program 22.0 (SSPS, Chica-go, IL, USA), using Chi square test to compare proportions. The margin of error for the study was plus or minus 5 percent points at a 95% level of confidence. A p-value of < 0.05 was considered statistically significant.

RESULTS

A total sample size of 50 Egyptian infant, attending the Alexandria University Children's Hospital at El-Shatby during the winter/spring season of 2013, and clinically diagnosed with acute bronchiolitis, was used in the present study.

Table (3, 4) and figures (11, 12) show both the age and sex distribution of the 50 infant patients included in the study; 44% of them were less than 3 months (peak age of infection), 34% were between 3 months to 6 months, whereas only 22% of the patients were in the age of 6 months or above. The median of age was 3.41 months and mean 4.42± 2.99 months. The majority of the studied patients (36/50) were males (72%).

Table (3): Age among all patients included in the study

	Min. – Max.	Mean ± SD.	Median
Age (months)	1.0 – 12.0	4.42 ± 2.99	3.41

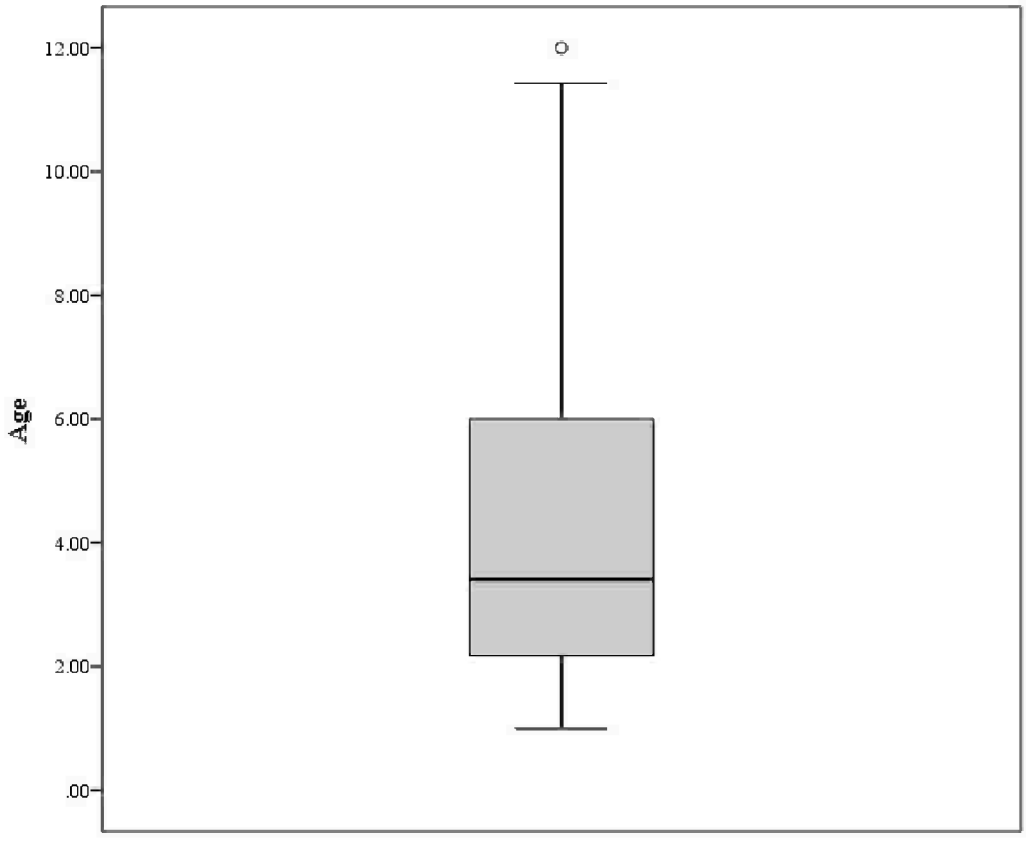


Figure (11): Age of all patients included in the study

Table (4): Sex distribution among all patients included in the study

Sex	No.	%
Female	14	28.0
Male	36	72.0

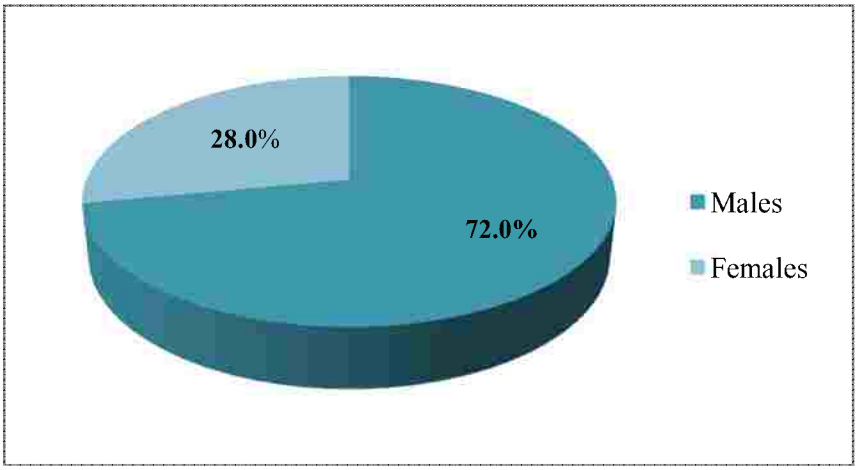


Figure (12): Chart of sex distribution among all studied patients

Samples from the 50 infant patients were collected during the winter/spring season of the year 2013 over a period of 3 months. As shown in table (5): 46% of the samples were collected in January, 40% were collected in February, and 14% were collected in March (Fig. 13).

Table (5): Distribution of studied cases during 3 months of the study

Month	No.	%
January	23	46.0
February	20	40.0
March	7	14.0
Total	50	100

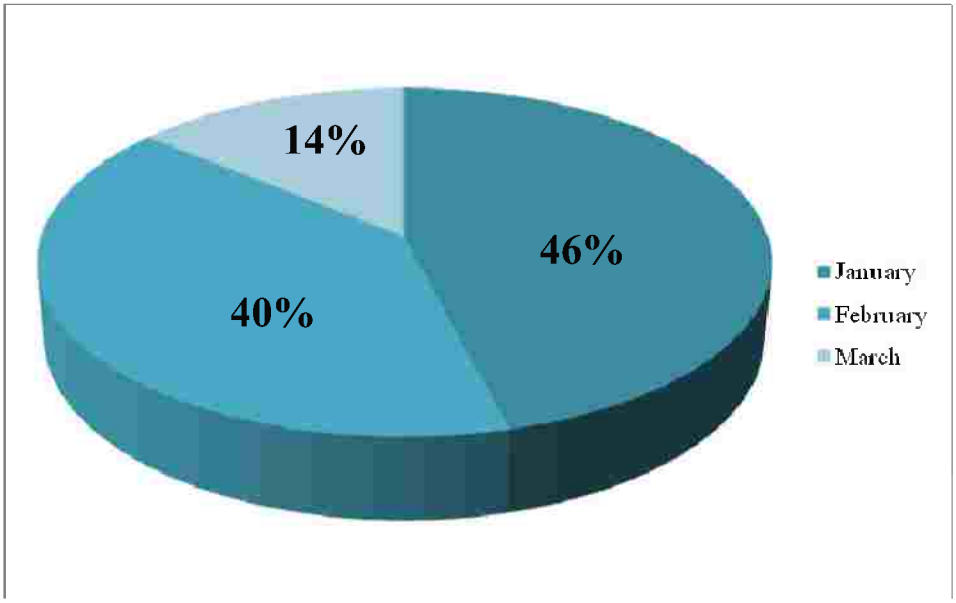


Figure (13): Chart of distribution of studied of studied cases during 3 months of the study

Table (6) shows the medical history of all enrolled patients (including the infant's birth weight, mode of delivery, previous Neonatal Intensive Care Unit [NICU] or hospital admission); 20% of all patients were born with a low birth weight. Fifty sex percent (56%) were delivered normally (vaginal), whereas 44% of the cases were delivered through a C-section. Twelve percent (12%) of infants were previously hospitalized at some time of their age. Twenty percent (20%) were admitted to a NICU.

Table (6): Medical history of all studied patients

History		No.	%
Birth Weight	**Normal**	40	80.0
	LBW*	10	20.0
Mode of Delivery	**Vaginal**	28	56.0
	C-section	22	44.0
Previous NICU Admission	**Yes**	10	20.0
	No	40	80.0
Previous Hospital Admission	**Yes**	6	12.0
	No	44	88.0

* LBW (or Low Birth Weight) is defined as a birth weight of a liveborn infant of less than 2.500 grams regardless of gestational age. [102]

Table (7) and figure (14) show the clinical and radiological findings observed among the 50 infant patients included in the study. Cough and wheezing were the most predominant findings, observed in 98% and 96% of the patients; respectively. A radiograph with a hyper-inflated chest (Fig. 23) was found in 94% of the cases. Fever and tachypnea were common too, observed in 72% and 62% of cases, respectively. Vomiting

and feeding difficulties were present in 56% and 54% of the cases, respectively. Twenty eight percent (28%) of the patients experienced rhinorrhea, and 18% had dyspnea. Sixteen percent (16%) of the cases were hypoxemic, whereas 12% had been suffering from grunting as a sign of respiratory distress. Diarrhea was found in only 6% of the cases. Otitis media was observed in only one case (2%).

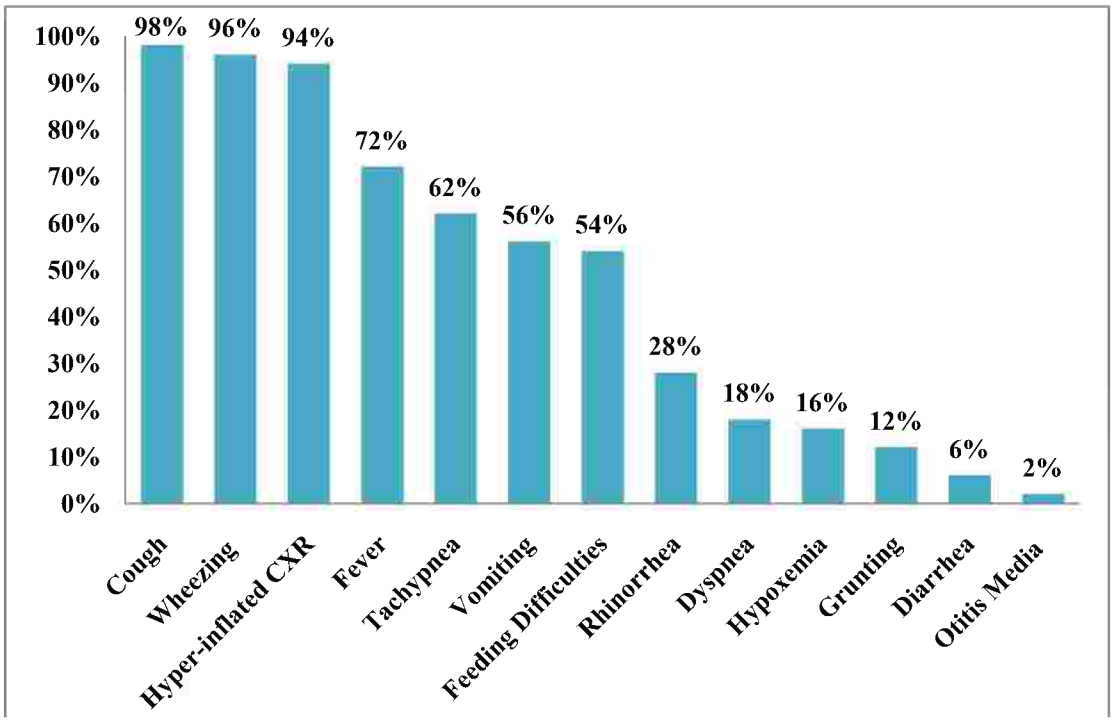
- Patients were presented by multiple symptotology.

Table (7): Clinical findings observed among all studied patients

Clinical presentation	No. of Cases	%
Cough	49	98.0
Wheezing	48	96.0
Hyper-inflated CXR	47	94.0
Fever*	36	72.0
Tachypnea*	31	62.0
Vomiting	28	56.0
Feeding Difficulties	27	54.0
Rhinorrhea	14	28.0
Dyspnea	9	18.0
Hypoxemia	8	16.0
Grunting	6	12.0
Diarrhea	3	6.0
Otitis Media	1	2.0

* Fever is a controlled increase in body temperature over the normal values for an individual .normal body temperature is considered to be ranged from (36_37.5°C) [103]

* Tachypnea is defined as an elevated respiratory rate. According to the WHO criteria, for newborns < 2 months, an infant is considered tachypneic if he has 60 breaths per minute. For infants > 2 months and younger than one year, tachypnea is considered if the child has 50 breaths per minute. [104]



(X-axis = clinical finding, Y-axis = percentage of cases)

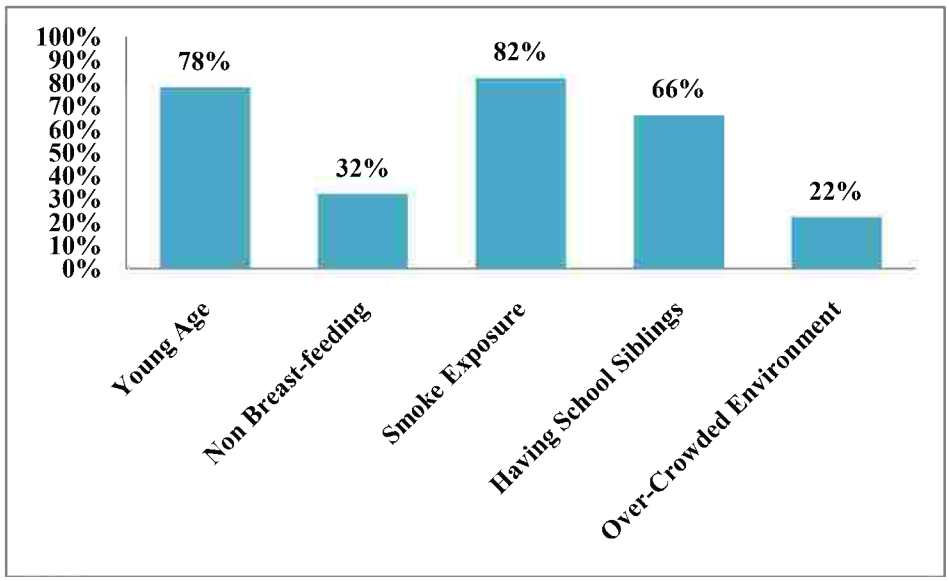
Figure (14): Chart of clinical findings observed among all studied patients

Table (8) and figure (15) show the different risk factors for acute bronchiolitis observed among all the studied patients; 78% of all patients were at young age (less than 6 months), 32% were bottle-feeders, 82% were exposed to tobacco smoke, 66% had school or child-care siblings, and 22% were living in a highly crowded environment.

Table (8): Risk factors for acute bronchiolitis observed among all studied patients

Risk Factor		No.	%
Age	Less than 6 months	39	78.0
	6 months or older	11	22.0
Mode of Feeding	Breast-feeding	34	68.0
	Bottle-feeding	16	32.0
Exposure to Smoking	Yes	41	82.0
	No	9	18.0
Having a sibiling attending School	Yes	33	66.0
	No	17	34.0
Crowdness*	Non-crowded (≤ 4 individuals)	7	14.0
	Crowded (5 - <10 individuals)	32	64.0
	Over-crowded (≥10 individuals)	11	22.0

* Determined by the approximate number of individuals living in an apartment consisting of four compartments. [105]



(X-axis = risk factor, Y-axis = percentage of cases)

Figure (15): Risk factors for acute bronchiolitis observed among all studied patients

According to the CBC results:

According to HB level nineteen infants were anemic (38%). The Min -Max was 7.6-14.8 with a mean of 10.86±1.51 gram/dl and normal HB level ≥12 gram/dl. According to platelets there are two infants were thrombocytopenic (4%).The Min -Max was 142-784 with a mean 430.36±160.89 and normal platelet count is (150000-450000). According to WBCS there were ten infants (20%) with high leukocytic count (normal leukocytic count up to 10000). The Min–Max was 4100-15500 with a mean 9762.6±2523.2. According to Monocytes the Min –max was 1.33-28% with a mean 7.64±4.97. According to Eosinophils the Min –Max was 0.20-18.20% with a mean 6.26±4.68. According to Basophils the Min – Max was 0.10-3.20% with a mean 1.12±0.77. According to Lymphocytes there were thirty infants (60%) were who had high lymphocytic count .The Min-Max was 2.70-77.0% with a mean 38.71±18.96 and normal lymphocytic count up to 35%.

Table (9): CBC value in the studied cases

	Min. – Max.	**Mean ± SD.**
HB (in gram)	7.60 – 14.80	10.86 ± 1.51
Platelets ($\times 10^3$)	142.0 – 784.0	430.36 ± 160.89
WBCS	4100.0 - 15500.0	9762.6 ± 2523.2
Monocytes %	1.33 – 28.0	7.64 ± 4.97
Eosinophils %	0.20 – 18.20	6.26 ± 4.68
Basophils%	0.10 – 3.20	1.12 ± 0.77
Lymphocytes%	2.70 – 77.0	38.71 ± 18.96

According to CRP there were seven infants (14%) who had high level. The Min – Max 1.10-30.40 with a mean 4.80±4.92 and normal value up to 5.

Table (10): C - reactive protein level in the studied cases

	Min. – Max.	Mean ± SD.
CRP	1.10 – 30.40	4.80 ± 4.92

Table (11) and figure (16) show the IMAGEN®Immunofluorescence Respiratory Screen Test results. This test was mainly used to qualitatively detect the viral antigens of any of the 7 most common respiratory viruses that could infect respiratory epithelia, and cause lower respiratory tract infections in young children. The assay was designed to test patient's nasopharyngeal samples for RSV, influenza A/B, parainfluenzatypes 1-3, and adenovirus. In this study, 76% of the cases were reported to be positive either to one or more of those seven mentioned viruses. The rest of cases (24%) were negative.

Table (11): Results of immunofluorescene test for respiratory viruses

Result	No.	%
Positive	38	76.0
Negative	12	24.0
Total	50	100

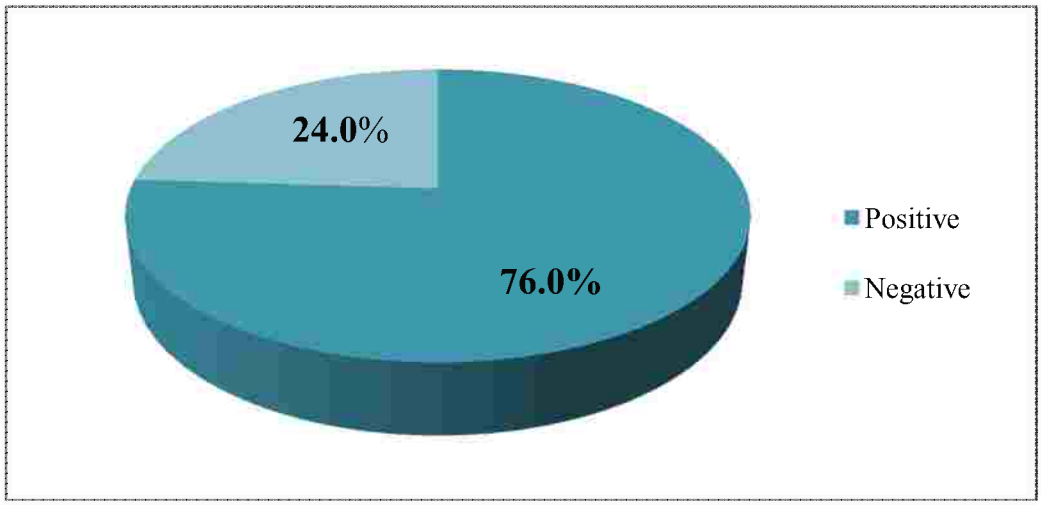


Figure (16): Results of immunofluorescene test for respiratory viruses

Figure (17) shows the IMAGEN®Immunofluorescence control slides. In the positive well (A), cells appeared with intracellular apple-green cytoplasmic and/or nuclear fluorescence contrasting against a background of red counterstained material. The negative well (B), on the other hand, showed cells with no intracellular apple-greenfluorescence. Only background red counter-staining was visible under fluorescence microscopy.

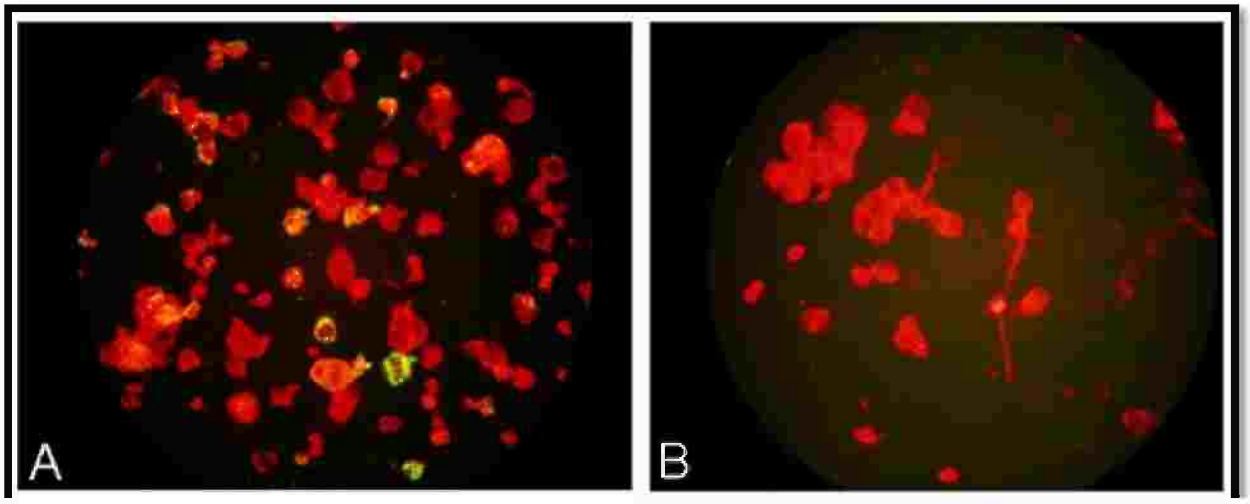


Figure (17): Positive and negative IMAGEN® immunofluorescence control slides (A = positive well, B = negative well)

Figure (18) shows a clinically positive IMAGEN® Immunofluorescence slide sample stained and prepared according to the instructions of the kit's manufacturer. As shown in figure (A), the cells in the fixed, stained specimen appeared to have the typical fluorescence pattern of the positive control slide shown in figure (17). A negative diagnosis (B) was made when fixed, stained specimens didn't exhibit this fluorescence with the screening reagent.

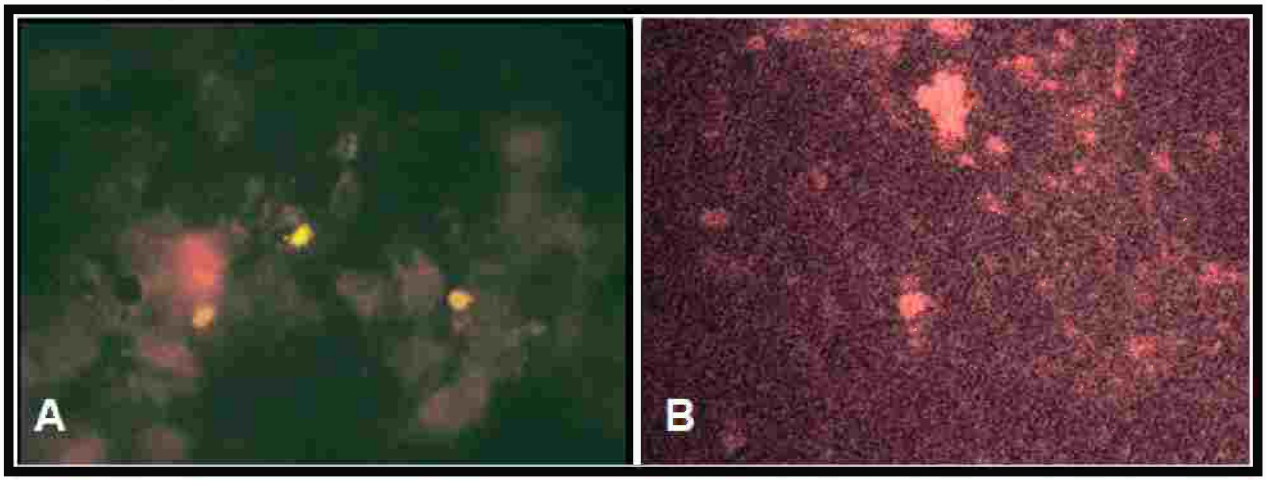


Figure (18): Clinical positive and negative imagen® immunofluorescence slide samples (A = Positive Sample, B = Negative Sample)

Table (12) and figure (19) show the PCR results for human metapneumovirus. Out of the 50 samples tested for the presence of hMPV nucleic acid, only 8 cases (16%) were reported to be positive using the advanced hMPVPrimerDesign™ genesig qPCR Detection Kit.

Table (12): Real-Time PCR results for human metapneumovirus of all cases

Result	No.	%
Positive	8	16.0
Negative	42	84.0
Total	50	100

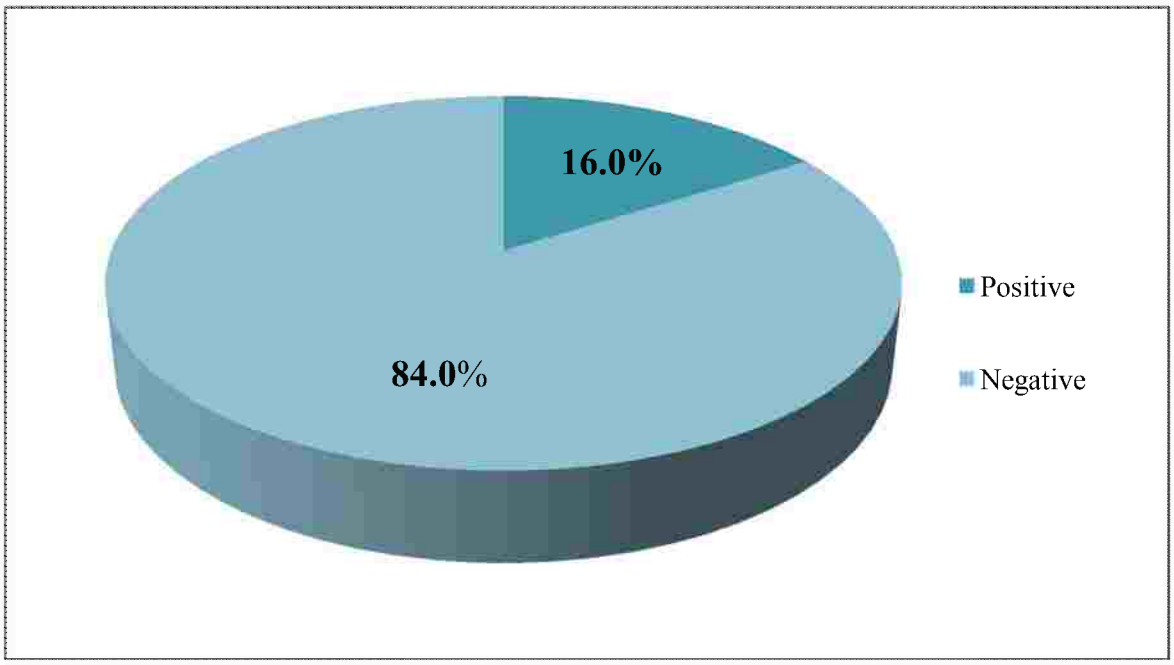


Figure (19): Chart of hMPV real-time PCR results

Figure (20) shows the real-time PCR amplification plot of three hMPV positive cases using the Applied Biosystems Real-Time OneStep™ PCR system. The first case (on the top) had a Ct (Threshold cycle) value of 26.The second case (in the middle) had a Ct value of 28. The third case (at the bottom) had an early Ct value of 16, indicating the presence of a high load of hMPV nucleic acid template in this sample.

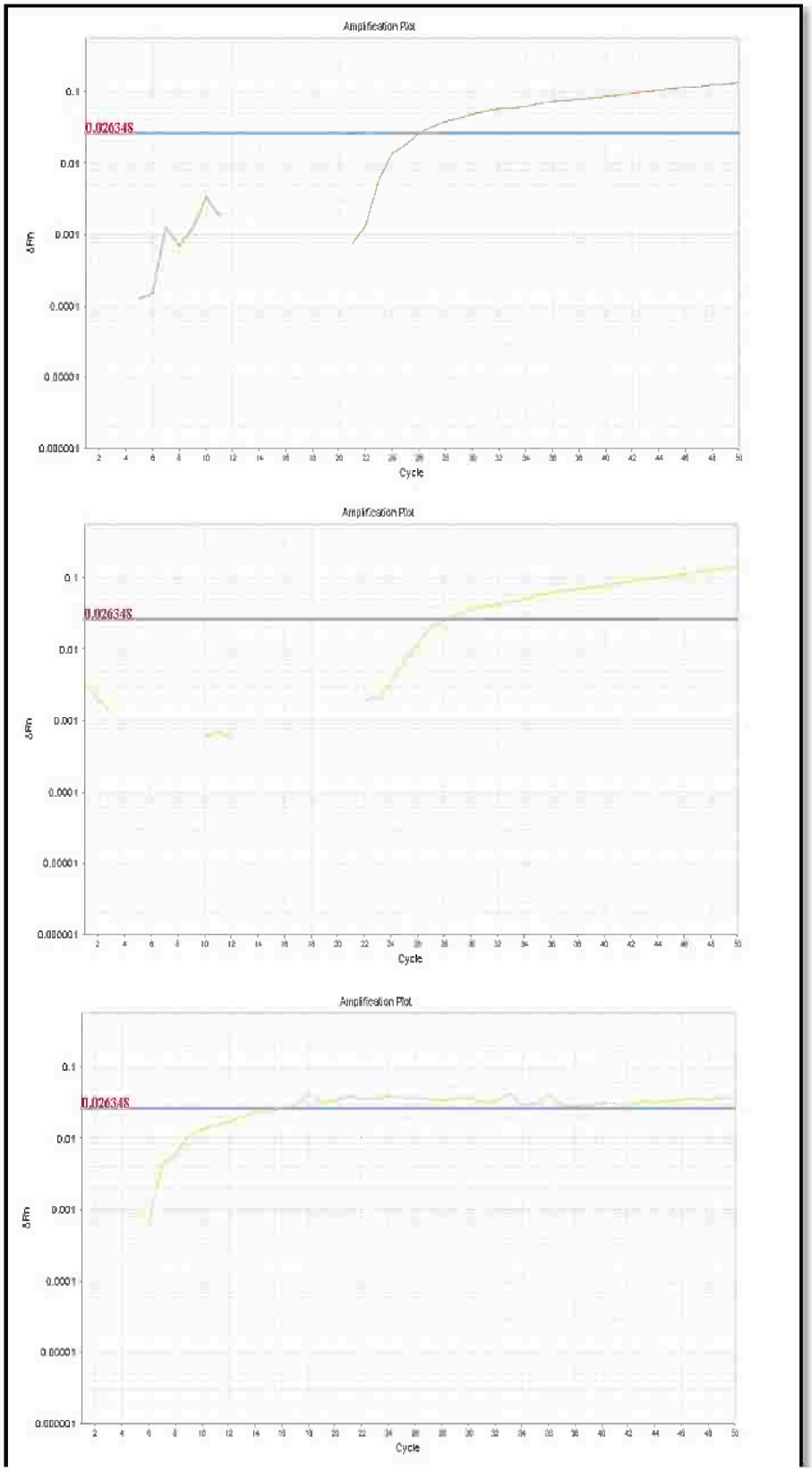


Figure (20): Real-time PCR Amplification Plots of 3 hMPV Positive Cases

Table (13) and figure (21) show the collective results for both of immunofluorescence and PCR assays. As shown in table:

A. **Thirty-two cases** (64%) were positive to RSV and/or influenza A/B, parainfluenza types 1-3 and adenovirus by immunofluorescence, but negative to hMPV by PCR.
B. **Six cases** (12%) were positive to hMPV by PCR, and by immunofluorescencefor at least one of the 7 respiratory viruses detected by the IMAGEN® respiratory screen kit (RSV, influenza A/B, parainfluenza types 1-3 and adenovirus). I.e. having a mixed infection.
C. Only **two cases** (4%) were positive only to hMPV by PCR, but negative to RSV, influenza A/B, parainfluenza types 1-3 and adenovirus by immunofluorescence.
D. **Ten cases** (20%) were negative to hMPV (by PCR), and to RSV, influenza A/B, para-influenza types 1-3 and adenovirus by immunofluorescence.

Table (13): Collective Results for Both Immunofluorescence and PCR Assays

		Immunofluorescence for RSV, Influenza A/B, Parainfluenza 1-3 and Adenovirus				Total	
		Positive		Negative			
		No.	%	No.	%	№	%
PCR Results for hMPV	Positive	6	12.0	2	4.0	8	16.0
	Negative	32	64.0	10	20.0	42	84.0
Total*		38	76.0	12	24.0	**50**	**100**

* Percentages were calculated from the total sample size (n=50), while other percentages were calculated from the total of each column

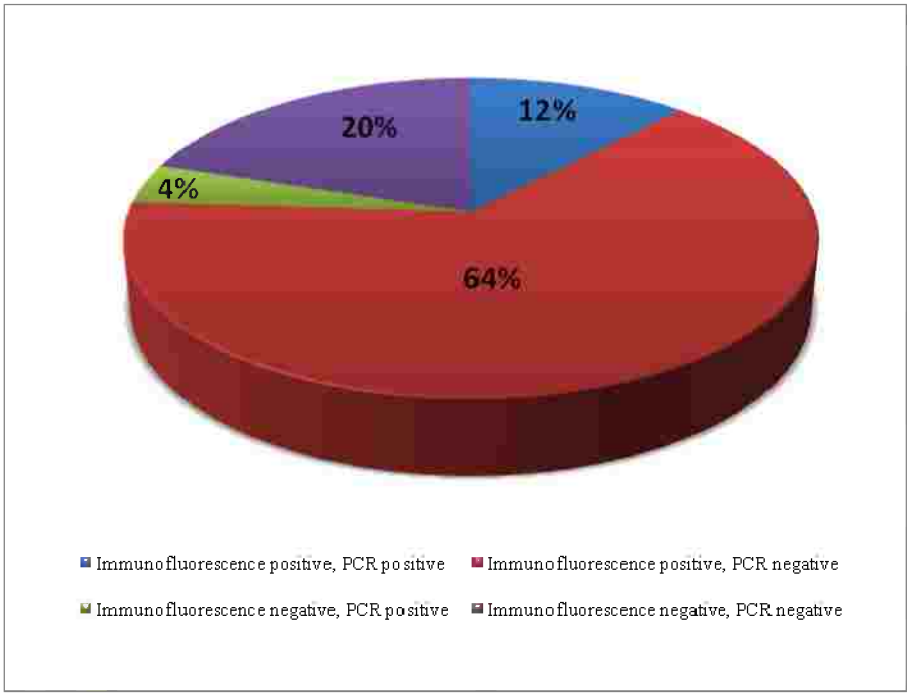


Figure (21): Chart of results for both immunofluorescence and pcr assays

Table (14) and figure (22) show the seasonality of human metapneumovirus infection in the study during the winter/spring season of 2013. As shown in table: 50% of the 8 hMPV-positive samples were detected in January (peak month of infection) and 17.39% from total infant with bronchiolitis, 37.5% of the 8 hMPV-positive samples were detected in February and 15% from total infant with bronchiolitis, and only 12.5% of the 8 hMPV-positive samples were detected in March and 16% from total infant with bronchiolitis.

Table (14): Seasonality of hMPV infection in the study

Month	No.	% of +ve 8 hMPV	Total of infected infant with bronchiolitis	% from total
January	4	50.0	23	17.39
February	3	37.5	20	15.0
March	1	12.5	7	14.3
Total	8	100	50	16.0

(X-axis = months, Y-axis = percentage of cases)

Figure (22): Chart of seasonality of hMPV infection in the study

Table (15) According to the clinical finding in patients with hMPV infection presented by cough were 8(100%), patient with respiratory viruses other than hMPV were 32 (100%) and patient negative to tested viruses were 9 (90%).

Patients with hMPV infection presented by wheezing were 8(100%), patient with respiratory viruses other than hMPV were 30 (93.7%) and patient negative to tested viruses were 10 (100%).

Patients with hMPV infection presented by fever were 8(100%), patient with respiratory viruses other than hMPV were 20 (62.5%) and patient negative to tested viruses were 8 (80%).

Patients with hMPV infection presented by feeding difficulties were 6 (75%), patient with respiratory viruses other than hMPV were 18 (56.2%) and patient negative to tested viruses were 3 (30%).

Patients with hMPV infection presented by dyspnea were 0(0%), patient with respiratory viruses other than hMPV were 9 (28.1%) and patient negative to tested viruses were 0 (0%).

Patients with hMPV infection presented by tachypnea were 5 (62.5%), patient with respiratory viruses other than hMPV were 19 (59.3%) and patient negative to tested viruses were 7 (70%).

Patients with hMPV infection presented by vomiting were 5 (62.5%), patient with respiratory viruses other than hMPV were 17 (53.1%) and patient negative to tested viruses were 6 (60%).

Patients with hMPV infection presented by rhinorrhea were 4 (50%), patient with respiratory viruses other than hMPV were 7 (21.8%) and patient negative to tested viruses were 3 (30%).

Patients with hMPV infection presented by hypoxemia were 2 (25%), patient with respiratory viruses other than hMPV were 6 (18.7%) and patient negative to tested viruses were 0 (0%).

Patients with hMPV infection presented by grunting were 2 (25%), patient with respiratory viruses other than hMPV were 1 (3.1%) and patient negative to tested viruses were 3 (30%).

Patients with hMPV infection presented by diarrhea were 1 (12.5%), patient with respiratory viruses other than hMPV were 1 (3.1%) and patient negative to tested viruses were 1 (10%).

Patients with hMPV infection presented by otitis media were 0 (0%), patient with respiratory viruses other than hMPV were 0 (0%) and patient negative to tested viruses were 1 (10%).

Patients with hMPV infection presented by hyper-inflated chest x-ray were 7(87.5%), patient with respiratory viruses other than hMPV were 31 (96.8%) and patient negative to tested viruses were 9 (90%).

According to above results it indicates no significant differences in clinical finding.

Table (15): Clinical Findings in Patients with Respiratory Viruses Other Than hMPV, Patients with hMPV, and Patients Negative to Any of the Tested Viruses in the Study

		Type of Viral Infection Detected						*p-value*	
		Respiratory viruses other than hMPV† (n=32)		hMPV with or without coinfection (n=8)		Negative to tested viruses (n=10)		$^{FE}p_1$	$^{FE}p_2$
		No.	%	No.	%	No.	%		
Clinical Finding	**Cough**	32	100	8	100	9	90.0	-	1.000
	Wheezing	30	93.7	8	100	10	100	1.000	-
	Fever	20	62.5	8	100	8	80.0	0.079	0.477
	Feeding Difficulties	18	56.2	6	75.0	3	30.0	0.439	0.153
	Dyspnea	9	28.1	0	0.0	0	0.0	0.162	-
	Tachypnea	19	59.3	5	62.5	7	70.0	0.720	1.000
	Vomiting	17	53.1	5	62.5	6	60.0	0.709	1.000
	Rhinorrhea	7	21.8	4	50.0	3	30.0	0.182	0.630
	Hypoxemia	6	18.7	2	25.0	0	0.0	0.650	0.183
	Grunting	1	3.1	2	25.0	3	30.0	0.096	1.000
	Diarrhea	1	3.1	1	12.5	1	10.0	0.356	1.000
	Otitis Media	0	0.0	0	0.0	1	10.0	-	1.000
Hyper-inflated CXR		31	96.8	7	87.5	9	90.0	0.364	1.000

† RSV and/ or Influenza A/B, Parainfluenzatypes (1- 3) or Adenovirus

$p_1$compareshMPV- positive patients with patientspositive to respiratory viruses other than hMPV.

p_2 compareshMPV- positive patients with patients negative to any of the tested viruses in the study.

Table (16) According to eight hMPV positive patient; the age less than 3 months were 3 (37.5%), between 3 months to 6 months were 2 (25%) and above 6 months were 3 (37.5%). There were six male (75%) and two female (25%).

According to risk factors, there was one (12.5%) was bottle fed, seven (87.5%) had exposure to smoking, 6 (75%) having asibling attending school and 2 (25%) who living in crowdness.

Table (16): Description of epidemiology and risk factor among 8 hMPV positive patients

Age	**(n = 8)**	**%**
< 3	3	37.5
3-6	2	25.0
≥ 6	3	37.5
Sex		
male	6	75.0
female	2	25.0
Risk factor		
Bottle feeding	1	12.5
Exposure to smoking	7	87.5
Having asibiling attending school	6	75.0
Crowdness	2	25.0