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Research Article

IDENTIFYING OF HUMAN METAPNEUMOVIRUS AND ITS PHENOTYPE AS A CAUSATIVE AGENTS OF PNEUMONIA IN CHILDREN

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ABSTRACT

Objectives: The present investigational study was aimed to detect and identify the genotypes of *Human metapneumovirus (hMPV*) and its phylogeny with respiratory *syncytial* viruses *(RSV*) that cause pulmonary inflammation.

Material and Methods: A total of 250 samples of patients who were clinically diagnosed respiratory tract illness were collected from Maternity and Children Hospital in Al Diwaniyah city, Iraq. The clinical samples were nasopharyngeal, nasal and throat swabs. The current study screened the presence of hMPV and RSV (A and B) genotypes from nasopharyngeal specimens of children aged from several days to 10 years old.

Results: The results revealed that 6% were infected with *hMPV*, 8% of respiratory *syncytial viruses type A (RSV-A*) and 14% of respiratory *syncytial virus's type B (RSV-B)* from children who are suffering from respiratory illness. Phylogenetic tree analysis of hMPV based on the partial sequences of the *fusion protein (F)* gene was used for genotyping and detection. The phylogenetic tree was constructed using maximum likelihood tree method in MEGA 6.0 version. The local *hMPV* isolates (S1) were closely related to NCBI-Blast *hMPV* genotype A1 (KM408076.1), the local *hMPV* isolates (S2, S3, and S5) were closely related to NCBI-Blast *hMPV* genotype B1 (KJ196323.1), and the local *hMPV* isolates (S4) were closely related to NCBI-Blast *hMPV* genotype B2 (JQ041689.1).

Conclusions: The prevalence rate of *hMPV* is less than RSV, and both subtypes of *hMPV*, A and B may exist and circulate in one season, and the predominant sublineage of *hMPV* shifts in progressive season.

Keywords: DNA, Sequencing, *Human metapneumovirus*, Phylogenetic tree, *Respiratory syncytial virus*

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INTRODUCTION

Human metapneumovirus (hMPV) infection was recognized and isolated in 2001 by specialists from the Netherlands, van den Hoogen *et al.* They observe this infection by investigating the cytopathic impact on cell society in tertiary monkey kidney cells [1] of nasopharyngeal aspirate from children who have got respiratory syndromes such as common cold, bronchiolitis, and asthma exacerbation [2-4].

The *hMPV* is an enveloped, single-strand, negative-sense RNA virus belonging to the family Paramyxoviridae, subfamily Pneumovirinae, and genus *metapneumovirus*. The *hMPV* has been classified into two genotypes: A and Nucleotides and amino acid sequence identities between the *hMPV* groups are 80% and 90%, respectively, each of them has two siblings: A1(NL 00-1 like), A2 (NL 93-1 like); B1(NL 99-1 like); and B2 (NL 94-1 like) [1]. The *hMPV* as other members of the *Paramyxoviridae* is a typically spherical, enveloped with a diameter of 150–200 nm. Another shape can be present such as filamentous with diameter 100–400 nm and pleomorphic. A lipid envelope that derived from the plasma membrane of infected cells surround the virus particles. The lipid layer of the *hMPV* is very vulnerable to organic chemical agents [5].

There are three virus glycoproteins on the viral envelope, glycoprotein G for attachment, F for fusion and small hydrophobic protein [6]. Glycoprotein G inside external carboxy-terminal components and inside internal amino acid, and while F protein with the amino terminus is located outside the virus particle and a short cytoplasmic carboxyl- terminal positioned inside part of the viral envelope. The G and F proteins composed of 10 to 14-nm spikes on the surface of virion, and it is strongest related to the *avian metapneumovirus type C,* formerly *turkey rhinotracheitis* virus, these two viruses have been classified into a distinct genus, metapneumovirus [2]. The genomic RNA of *hMPV* associated with the viral glycoprotein (N), large (L) proteins, and the phosphoprotein (P) to produce the "helical nucleocapsid" which is situated inside M-protein that produces a linkage between the nucleocapsid and envelope of the virion. The M2-1 transcriptional enhancer protein is also thought to be related to the nucleocapsid [7]. The universal circulation forms of *hMPV* are inconstant and sophisticated. Recently, the epidemics of *hMPV* are extremely restricted and public-dependent despite the frequency of *hMPV* infection differ annually. Both genotypes strains, all sub-genotypes, and lineages may stream concomitantly in a particular position during a particular period [8,9]. In addition, the worldwide and localized *hMPV* epidemics can differ yearly which dominating circulating strains. Global effective vaccine of *hMPV* required understanding the genetic heterogeneity of the strain [10]. Hence, the present study was aimed to detect, identify the genotype *hMPV* and its phylogeny with *respiratory syncytial viruses (RSV*) that cause pulmonary inflammation.

MATERIALS AND METHODS

Samples collection

Informed consent was obtained from all patients, and the project was approved by the College of Medicine, University of Al-Qadisiyah Ethics Committee.

The current study was carried out in College of Medicine laboratories, University of Al-Qadisiyah, Iraq, in the period from December 2014 to December 2015. 230 samples of patients who were clinically diagnosed respiratory tract illness were collected from Maternity and Children hospital in Al Diwaniyah city, Iraq. Information of each patient was

recorded through a questionnaire format which has been constructed by the supervisors and pediatricians. The questionnaire included the name of the patient, age, gender and the major clinical features such as fever, cough, vomiting, and nasal discharge (rhinorrhea illness). The clinical samples were nasopharyngeal, nasal and throat swabs. The samples were collected in vials containing viral transport media which prepared for this purpose, then transported to the laboratory for storage at −70°C until use.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Viral RNA was extracted from frozen nasopharyngeal swabs using AccuZolTM total RNA extraction kit (Bioneer, South Korea) and prepared based on manufacturer's instructions. To duplicate a piece of the "open reading frames (ORFs)" of the *F. gene*, the RT-qPCR set and used. The primers and probe were designed in this study using complete sequence of *nucleoprotein gene RSV-A* (GenBank: KF973340.1), *RSV-B* (GenBank: KF893260.1), and *hPMV* (GenBank: KF891365.1) from NCBI-GenBank and Primer3 plus design. The primers were supplied by Bioneer Company, South Korea, as revealed in Table 1.

RT-qPCR was performed for detection of *respiratory syncytial virus type An (RSV-A), respiratory syncytial virus type B (RSV-B) and human metapneumo*virus based on *nucleoprotein* gene and this technique were carried out according to the method described by manufacturer's instructions. RT-PCR master mix was prepared according to manufacturer's instructions of Bioneer company, South Korea, as shown in Table 2.

The RT-PCR master mix reaction constituents that stated in Table 2 were supplemented into RT-PCR tube containing (8 wells strips tubes which containing Rocketscript reverse transcriptase and TaqMan probe premix). Then, all strips tubes vortexing for mixing the components and centrifuged (3000 rpm for 3 min in an Existing centrifuge), next to that moved into real-time PCR thermocycler. The conditions of real-time PCR thermocycler were set based on the temperature of primer annealing and RT-PCR TaqMan instructions of kit as revealed in Table 3.

The data of RT-qPCR of target and housekeeping genes were examined by the comparative quantification gene expression levels (fold change) that designated by Livak and Schmittgen (2001). The DNA sequencing was performed for genotyping of *hMPV* based on F-fusion gene. The PCR product of hMPV F-gene (428 bp) was refined from agarose gel using the EZ-10 Spin column DNA Gel Extraction kit (Bio Basic, Canada). After that, the refined F-gene was directed to Bioneer company (South Korea) for conduction the DNA sequencing.

Phylogenetic tree

Consensus sequences were generated using MEGA 6.0, multiple alignment analysis tools. The sequences nucleotide belongs to normal species from the both "*hMPV* genotypes and sub-lineages" were gained from "NCBI-GenBank *human metapneumovirus"* based ClustalW alignment analysis of *fusion protein (F) gene* and used to constitute both phylogeny and alignments.

RESULTS AND DISCUSSION

The results revealed that only 7% were encountered with the hMPV, 8% and 14% were detected with RSV-A and B genotypes, respectively [Fig.s 1-3]. There are several studies revealed different results of detected of hMPV, the lowest prevalence has been reported in Cambodia, 1.7%, and 2.6%% in the USA and the highest prevalence rate reported was 54.4% in Al-Ahwaz province [11-13] while the prevalence rate of RSV was 29.3% in infants with median age 13.5 months as in the study of [14].

In the subsequent study, it deliberated certain the genetic variation (genotyping) of human metapneumovirus to divulge the only five

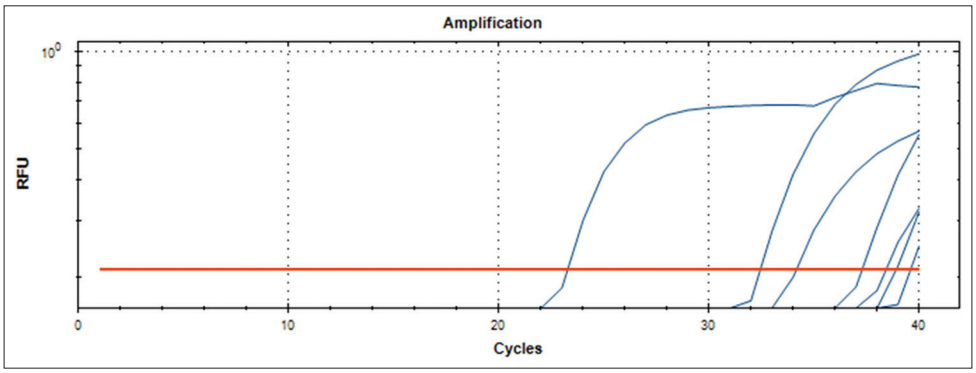

Fig. 1: Real-time polymerase chain reaction amplification plot of positive samples for human metapneumovirus based nucleoprotein gene primers and probe (FAM) dye of nasopharyngeal swabs, where the positive samples were shown positive amplification ranging from 23.24 into 39.56 threshold cycle (CT)

Table 1: Primers of F *gene* used in this study

Primer	Sequence("5→3")		Amplicon size
RSV-A primer	F	TGCAGGGCAAGTGATGTTAC	86bp
	R	TTTCTGCTTGCACACTAGCG	
RSV-A probe	VIC-GGTGGGGAGTCTTAGCAAAATCAGTT-BHQ-1		
SV-B	F	TGTGCACTTTGGCATTGCAC	
	R	TTACTTGCCCTGAACCATAGG C	101bp
RSV-B probe	NED-TCCACAAGAGGGGGTAGTAGAGTTGA- BHQ-1		
HMPV primer	F	AGAAACTCAGGCAGTGAAGTC C	130bp
	R	TCTCTTCCACCCAGCTTTTCTC	
HMPV probe			

F: Forward; R: Reverse, HMPV: *Human metapneumovirus*, RSV: Respiratory *syncytial* viruses, RSV-A: Respiratory *syncytial* viruses-Type A, RSV-B: Respiratory syncytial viruses-Type B

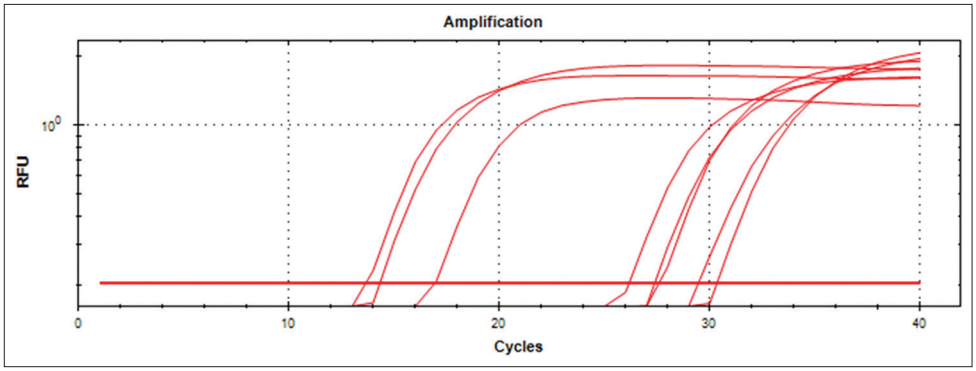

Fig. 2: Real-time polymerase chain reaction amplification plot of positive samples for Human respiratory syncytial virus type A based nucleoprotein gene primers and probe (VIC) dye in nasopharyngeal swabs samples, where the positive samples were shown positive amplification ranging from 13.79 to 30.28 threshold cycle (CT)

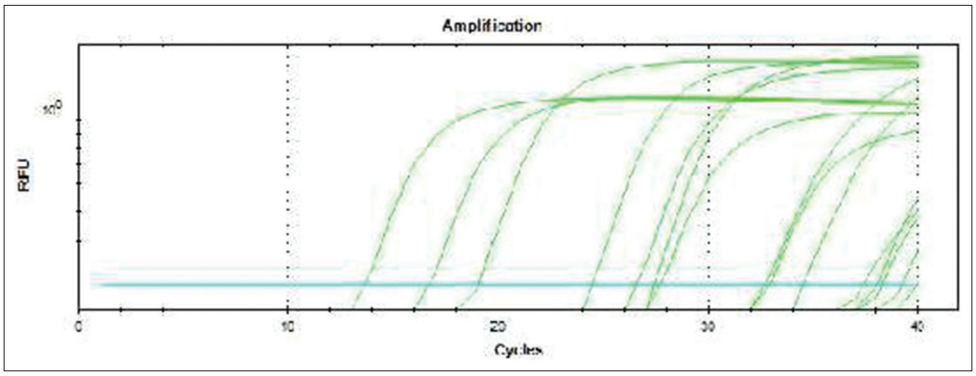

Fig. 3: Real-time polymerase chain reaction amplification plot of positive samples for Human respiratory syncytial virus type B based nucleoprotein gene primers and probe (NED) dye in the nasopharyngeal swab samples where the positive samples were shown positive amplification ranging from 13.72 to 39.94 threshold cycle (CT)

Table 2: Components of PCR master mix

RT-PCR master mix	Volume (μL)
Total RNA template	5
RSV-A, RSV-B, HMV Forward primer (20 pmol)	2.5
RSV-A, RSV-B, HMV Reverse primer (20 pmol)	2.5
RSV-A, RSV-B, HMV probe (25pmol)	2.5
DEPC water	37.5
Total	50

RT-PCR: Reverse transcription polymerase chain reaction, RSV-A: Respiratory *syncytial* viruses-Type A, RSV-B: Respiratory *syncytial* viruses-Type B

Table 3: RT-PCR thermocycler conditions

Step	Conditions	Cycle
Reverse transcriptase	50°C , 15 min	1
Pre-denaturation	95°C, 5 min	1
Denaturation	95°C, 20 s	
Annealing/extension		
Detection (scan)	60°C, 30 s	

RT-PCR: Reverse transcription polymerase chain reaction

local isolates and align with the reference isolates that have been brought from NCBI-GenBank *human metapneumovirus* and revealed distinctiveness of particular isolates, beyond that, this section will focus on the comparison with other studies in different countries and different places, and they will have known the predominant strain (genotype) of human metapneumovirus, at the end of these papers would be doing to determine the cause of the predominance local isolates [Fig. 4].

The aforementioned sequence alignment of the partial *fusion protein (F)* gene sequence of local isolates of *hMPV*, the *fusion protein (F)* gene has several characteristics that made it the best gene to analyze the genotyping of *hMPV*, first of all, the *F* gene is more conserved than other genes, rather than it encodes a 539 - amino acids, protein, this length of putative fusion protein ORF is neither so short such as M2-2 protein, 71, nor so long such as L protein which is 2005 in length; therefore, the analysis comparison is adequate to determine the genetic diversity of *hMPV*, the analysis sequence of *hMPV* relies on sequencing of the otherwise genes such as *N, M, G,* and *L* is the genotype grouping and is concordant anyhow of which is studied [5,15,16]. In addition, there is another important feature, the F-protein is capable of provoking the immune response and producing a potent immunoglobulin that neutralizes the virus and regardless of majorly homologous, the cross-productivity can occur [17,18]. However, depending on genetic sequencing of *hMPV* and construction of phylogenetic tree [Table 4], there are two main genotypes of *hMPV* which labeled *hMPV-A* and *hMPV-B*, likewise, these genotypes also separated into two sublineages as A1 and A2 so B1 and B2. In the current study, nucleotides sequencing was gained from 5 (5/100, 5%) *hMPV* positive samples. Amplified products are elected to concede to the partial genome sequencing of the *F* gene for determination of the local isolates of *hMPV* subtypes, the income of these sequencing is two genotypes A and B, *HPV*) genotype A1 is found in 1/5, 20%, A2 is not existing in the current study, and B1 is present in 3/5, 60% and B2 1/5, 20%, additionally, the homology of local current isolates was 100% of *hMPV-S1, hMPV-S3*, at the nucleotides sequencing level with the KM408076.1 and KJI96323.1, respectively, and 99% of hMPV-S4 with JQ041689.1 strain, three of five *hMPV-S2, S3*, and *hMPV* were identical (100%). Therefore, the

Table 4: DNA homology sequence identity using NCBI- BLAST alignment tool

Local *hMPV*	Homology sequence identity (%)			
	***hMPV* Genotype A1 (KM408076.1)**	***hMPV* Genotype A2 (KJ196309.1)**	***hMPV* Genotype B1 (KJ196323.1)**	***hMPV* Genotype B2 (JQ041689.1)**
hMPV-S1	100%	91%	93%	89%
hMPV-S2	86%	86%	100%	94%
hMPV-S3	86%	86%	100%	94%
hMPV-S4	86%	85%	93%	99%
hMPV-S5	86%	86%	100%	94%

HMPV: *Human metapneumovirus*

Fig. 4: Multiple sequence alignment analysis of the partial *fusion protein (F) gene* sequence in local *human metapneumovirus* isolates with for N C B I -GenBank *human metapneumovirus* based C l u s t a l W alignment analysis by using (MEGA 6 . 0 , m u l t i p l e alignment analysis tools). The multiple alignment analysis similarities (*) and differences in *fusion protein (F)* gene nucleotide sequences

genotype B is predominant (80%), the predominance rate of *hMPV* is problematic and dynamic, *MPV's* strains unlikely the *influenza* viruses which responsible for a global pandemic due to exhibits swift genetic evolution through the antigenic shift and antigenic drift, while *hMPV* reveals 2-3 worldwide spread annually. The outbreak of *hMPV* demonstrates to be a local occurrence [19], however, RNA viruses reveal a genetic mutation frequently because of this RNA virus absence of proofreading capability of RNA-dependent RNA polymerase [20].

In comparison with other studies that have been accomplished in every continent, have shown distinctive proportional than current study, *hMPV* strains display fluctuation starting from one population to another, additionally, pick *hMPV* strains in one population may be likely to strains that observed in somewhere else in various seasons. Nor'E *et al.* have shown that the Malaysian isolates of *hMPV* grouped predominantly into two sublineages, A2b (54%) and B1 (42%), of the same study, B1 is predominant in 2012, makeup 9/11 (81.8%) of the strains. Another study as that done by Liat *et al.*, have displayed only one subgenotype A2 was obtainable, furthermore, another study that's done by Qaisy *et al.*, in Jorden, the lineage A was detected in 93%, while 28.6% with the lineage B. Although these previous studies differ from the current study in recognition and analyzing of the predominant genotype, there are anther studies have revealed the similar genotype, which has recognized in this study, as the research that has achieved in Upper Egypt by Mohamed *et al.* which displayed the subgroup B2 was predominant. Similar to this result, in Cambodia between 2007 and 2008, [10] they observed that the sublineage B2 strain is predominant. However, reemerged and recirculation of genotype and sublineage have been observed to differ every year, which are substituted each 1 to 3 years inside a particular community, and it is believed to happen to rely on particular improvement of acquired immunity to the infected strain of the predominant coursing genotype [25-28].

The phylogenetic tree was constructed using Maximum Likelihood tree method in MEGA 6.0 version. The local HMV isolates (S1) showed closed related to NCBI-Blast *human metapneumovirus* genotype A1 (KM408076.1), the local *hMPV* isolates (S2, S3, and S5) were closely related to NCBI- Blast *human metapneumovirus* genotype B1 (KJ196323.1), and the local *hMPV* isolates (S4) were closely related to NCBI-Blast *human metapneumovirus* genotype B2 (JQ041689.1) [29].

CONCLUSION

The prevalence rate of *hMPV* is less than a respiratory syncytial virus, and both subtypes of *hMPV*, A and B may exist and circulate in one season, and the predominant sublineage of *hMPV* shifts in progressive season. The genotypes of *hMPV*, A1, A2, B1, and B2 are prevalently varied, and they do reveal different to other isolates of other places in the world, these genetic diversities of *hMPV* strains produces recurrent infection and subterfuge a challenge for future vaccine development.

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