Molecular study of human metapneumovirus among patients with respiratory tract infections in Diyala province

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Abstract:
Human Metapneumovirus (HMPV) is a paramyxovirus that is a common cause of bronchiolitis and pneumonia in children, elderly and immunocompromised patients. HMPV is responsible for 5% - 10% of children hospitalized for acute respiratory tract infections (RTIs). Molecular detection and genotyping of HMPV from patients with RTIs in Diyala province and exploring its association with the clinical picture. This cross-sectional study was conducted in the Diyala province, throughout two seasons; from January to May and from November to December/2018. A total of 323 patients suspected of having RTIs of different ages were enrolled. Data of participants including socio-demographic and clinical observations were collected by using a questionnaire specially designed for this purpose. Three types of respiratory samples were collected including throat, nasal and nasopharyngeal swabs. Real-Time PCR assay was used for molecular detection of HMPV, and two other sets of PCR primers for Attachment (G) and Fusion (F) Human Metapneumovirus genes have been used in the conventional PCR amplification in order to get PCR products used in the sequencing method for genotyping of the virus and phylogenetic tree analysis. Human privacy was respected by obtaining verbal consent. Statistical analysis of data was carried out using the statistical package (SPSS version 25). Statistical significance was considered if the P-value was equal or less than 0.05. Molecularly, the results revealed that out of 323 specimens, 30 (9.3%) specimens were positive for HMPV, with significantly higher in season II versus season I (93.3% vs 6.7%, P= 0.0001), particularly during November (50%, P= 0.0001). Furthermore, a significantly highest HMPV positivity rate was detected among nasal sinus swabs (96.7%, P= 0.0001). Additionally, the positivity rate was insignificantly higher in up to 5 years old children (P= 0.060). Clinically, cough (73.3%) and Bronchiolitis/bronchitis (86.7%) were significantly associated with HMPV positivity rate (P= 0.006 and P= 0.001) respectively. Genotypically, 15 hMPV positive samples were showed good (G) gene sequences that were submitted to NCBI, and all the Iraqi strains were appeared to be clustered within the sub-lineage B2 only. In conclusion, human Metapneumovirus was the mostly detected respiratory viral pathogen among less than 5 years of Iraqi children complaining of respiratory tract infection with phylogenetically predominance of sub-lineage B2. The seasonal variation of HMPV can be observed in this study. Emerging HMPV strains are continually evolving.

Keywords: Human metapneumovirus, viral respiratory tract infections, Diyala

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Introduction:
Globally, respiratory viral infections (RVIs) are an important cause of morbidity and mortality affecting peoples of all ages particularly, infants, young children, the elderly, as well as immunocompromised individuals [1,2]. Upper respiratory tract (URT) and lower respiratory tract (LRT) are frequently caused by a wide spectrum of viruses causing various clinical syndromes with variable outcomes ranging from common colds, pharyngitis, croup (laryngotracheobronchitis), otitis media, bronchiolitis, and viral pneumonia [3]. The
most important RVIs are influenza viruses type A and B, Human Respiratory Syncytial Virus (HRSV), Human Parainfluenza viruses (hPIVs), and Human Adenoviruses (HADVs), plus newly discovered human respiratory viruses including HMPV, Severe Acute Respiratory Syndrome Coronavirus (SARS), Middle East Respiratory Coronaviruses (MER-CoV), Human Bocavirus, and Human Rhinovirus. Among these viruses is the HMPV which is an emerging respiratory pathogen that was firstly described in 2001. Genetic analysis revealed that HMPV is quite similar to the respiratory viruses of the Pneumoviridae family. The HMPV is an important viral agent associated with severe bronchiolitis and pneumonia in children, and its symptoms are indistinguishable from those caused by a human respiratory syncytial virus. Initial infection with HMPV usually occurs during early childhood, but re-infections are common throughout life. Phylogenetic analysis of the nucleotide sequences indicated that there were two genetic groups of hMPV. Furthermore, each group is subdivided into two subgroups. HMPV encodes three surface proteins, F, G and SH proteins. The majority of antibodies to HMPV in serum were against F protein, which mediates cross-group neutralization and protection. The incidence of hMPV-associated respiratory infection was estimated at 5% to 10% in children and 2-4% in adults. In China, the HMPV infections were mainly caused by the B1 lineage which is phylogenetically related to strains from Japan.

Material and methods:
This cross-sectional study was conducted in the Diyala province, throughout two seasons; from January to May/2018 and from November to December/2018. The study samples were collected at Baquba Teaching Hospital and Al-Batool Teaching Hospital for Maternity and Children. A total of 323 patients from those complaining RTIs were enrolled; 185 patients were in the first season and 138 in the second season. The ultimate diagnosis was based on clinical, X-ray and laboratory findings, these cases were categorized as follows: Upper respiratory tract infections (URTIs); Tonsillitis, otitis media, coryza, rhinorrhea, pharyngitis, acute laryngitis, sinusitis and sore throat, lower respiratory tract infections (LRTIs); Includes bronchiolitis, bronchitis, pneumonia, influenza-like illness, dyspnea/tachypnea, cough, wheezing and chronic respiratory conditions, such as asthma exacerbation.

Clinical and socio-demographic data of participants were collected by using a questionnaire specially designed for this purpose. Three types of respiratory samples were collected including throat, nasal and nasopharyngeal swabs using a standard ∑-Virocult, UK swabs, after swabbing, swab plastic applicator shaft was broken off from the breaking point, and each cellular foam-bud tip (absorbent) swab was placed into the vial containing 1 milliliter of viral transport medium (mwe / UK and E.coli Ltd. / Russia) and transported by cooled box to the blood bank department in the hospital and stored at (-80°C) until molecular analysis was carried out. In this study, real-time RT-PCR hybridization-fluorescence amplification was used to multiplex detected and identified specific nucleic acid fragments for the seven RNA/DNA viruses, HRSV and HMPV among them. Human privacy was respected by obtaining verbal consent. Moreover, the study was approved by the Research Ethics Committee in the Diyala Directory of Health.

Statistical analysis of data was carried out using the statistical package (SPSS- version 25). Data were presented in simple measures of frequency, percentage, mean, standard deviation, and range (minimum-maximum values). The significance of the difference of means (quantitative data) was tested using Students-t-test for the difference between two independent means or ANOVA tests for difference among more than two independent means. The significance of the difference in percentages (qualitative data) was tested using the Pearson Chi-square test. Statistical significance was considered whenever the P-value was equal or less than 0.05.

Primers:
All primers and probes used in this study were selected from one line published literature after careful and thorough review. The suitability and specificity of these primers and probes have been confirmed in the NCBI primer basic local alignment search tool (BLAST). Two sets of PCR primers for Attachment (G) and Fusion (F) Human Metapneumovirus genes have been used in the conventional PCR amplification in order to get PCR products used in the sequencing method for genotyping of the virus and phylogenetic tree analysis. Primers were provided by (IDT Company / USA), table (1).

<table>
<thead>
<tr>
<th>Table (1): set of primers used for conventional and RT-PCR for hMPV.</th>
</tr>
</thead>
</table>

The RNA of hMPV from different swabs was extracted by a commercial kit (Zymo Research/USA) according to the manufacturer’s protocol (The Quick-RNA™ Viral RNA Extraction kit). The RNA was washed and eluted with DNase/RNase-free water, and then it is suitable for subsequent procedures including RT-PCR and Conventional PCR.

**Results:**
The results revealed that out of 323 specimens, 30 (9.3%) specimens were positive for hMPV; with an obviously significant increase of the infection rate during season II versus season I (93.3% Vs 6.7%, P=0.0001), particularly during November (50%, P=0.0001), table (2). Among the three types of respiratory specimens, it is clearly evident that the highest hMPV positivity rate was detected among nasal sinus swabs with a significant difference (96.7%, P=0.0001), table (3).

<table>
<thead>
<tr>
<th>Type of virus</th>
<th>Status</th>
<th>Total</th>
<th>Season I</th>
<th>Season II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>HMPV</td>
<td>Positive</td>
<td>30</td>
<td>9.3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>293</td>
<td>90.7</td>
<td>183</td>
</tr>
</tbody>
</table>

*Significant difference between proportions using Pearson Chi-square test at 0.05 levels.

**Table (3):** The hMPV positivity rate by type of specimens.

<table>
<thead>
<tr>
<th>Type of specimen</th>
<th>HMPV Positive</th>
<th>HMPV Negative</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasopharyngeal swabs</td>
<td>1</td>
<td>70</td>
<td>0.0001*</td>
</tr>
<tr>
<td>Nasal sinus swabs</td>
<td>29</td>
<td>132</td>
<td>45.1</td>
</tr>
<tr>
<td>Throat swabs</td>
<td>-</td>
<td>91</td>
<td>31.1</td>
</tr>
</tbody>
</table>

*Significant difference between proportions using Pearson Chi-square test at 0.05 levels.

Furthermore, data presented in table (4) clearly revealed that the positivity rate was highest in less than 5 years old children (86.7%). However, the difference was failed to reach the levels of statistical significance (P=0.060).

**Table (4):** The hMPV positivity rate by age groups.

<table>
<thead>
<tr>
<th>Age groups (Yrs)</th>
<th>HMPV positive</th>
<th>HMPV Negative</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>&lt;5</td>
<td>26</td>
<td>162</td>
<td>55.3</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>44</td>
<td>15.0</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>30</td>
<td>10.2</td>
</tr>
<tr>
<td>20</td>
<td>-</td>
<td>13</td>
<td>4.4</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
<td>11</td>
<td>3.8</td>
</tr>
<tr>
<td>40</td>
<td>-</td>
<td>9</td>
<td>3.1</td>
</tr>
<tr>
<td>50</td>
<td>-</td>
<td>12</td>
<td>4.1</td>
</tr>
<tr>
<td>=&gt;60</td>
<td>-</td>
<td>12</td>
<td>4.1</td>
</tr>
</tbody>
</table>

*Insignificant difference between proportions using Pearson Chi-square at 0.05 levels.
The results also showed that the hMPV positivity rate was equally (50%) distributed between both sexes with an insignificant difference (P=0.579). Clinically, the results revealed that cough was significantly associated with hMPV positivity (73.3% Vs 26.7%, P= 0.006). Similarly, Bronchiolitis/bronchitis were significant association with HMPV infection (86.7% vs 13.1%, P= 0.001). On the contrary, Fever/chill, pneumonitis, Pharyngitis and chronic obstructive pulmonary disease had a significantly negative association with hMPV positivity (30% vs 70%, P= 0.542, 13.3% vs 86.7, P= 0.733, 10% vs 90%, P=0.034) respectively.

Figure (1): Real-Time PCR amplification log plot showed cycles of HMPV positive results ranged from CT:20.4 to CT:26.9.

In spite of the detection of the hMPV F gene, none of these showed good sequences analysis results, while (15) of (G) gene samples showed good sequences which were submitted to NCBI with accession numbers:

<table>
<thead>
<tr>
<th>BankIt2243972 seq1</th>
<th>MN178606</th>
</tr>
</thead>
<tbody>
<tr>
<td>BankIt2243972 seq2</td>
<td>MN178607</td>
</tr>
<tr>
<td>BankIt2243972 seq3</td>
<td>MN178608</td>
</tr>
<tr>
<td>BankIt2243972 seq4</td>
<td>MN178609</td>
</tr>
<tr>
<td>BankIt2243972 seq5</td>
<td>MN178610</td>
</tr>
<tr>
<td>BankIt2243972 seq6</td>
<td>MN178611</td>
</tr>
<tr>
<td>BankIt2243972 seq7</td>
<td>MN178612</td>
</tr>
<tr>
<td>BankIt2243972 seq8</td>
<td>MN178613</td>
</tr>
<tr>
<td>BankIt2243972 seq9</td>
<td>MN178614</td>
</tr>
<tr>
<td>BankIt2243972 seq10</td>
<td>MN178615</td>
</tr>
<tr>
<td>BankIt2243972 seq11</td>
<td>MN178616</td>
</tr>
<tr>
<td>BankIt2243972 seq12</td>
<td>MN178617</td>
</tr>
<tr>
<td>BankIt2243972 seq13</td>
<td>MN178618</td>
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<tr>
<td>BankIt2243972 seq14</td>
<td>MN178619</td>
</tr>
<tr>
<td>BankIt2243972 seq15</td>
<td>MN178620</td>
</tr>
</tbody>
</table>

Phylogenetically, it was noted that all the Iraqi strains clustered within the sub-lineage B2 only with high homologies (98%) have been observed with the reference NCBI genomic DNA sequences of KX829167.1, which belongs to the Spanish HMPV genotype B, sub-lineages B2.
Discussion:
The importance of this study surely emerges from its uniqueness in Diyala province and its comprehensiveness as it covers the most common viruses causing RTIs that are detected by recent molecular techniques. In Iraq, annual specific precautions had usually adopted against RTIs during the winter season that necessitates preparedness of health authorities before adequate time. Among exacerbating factors is the family crowdedness plus the majority of Iraqi families still use the kerosene heaters as a home warming system.

These heaters are sharply elevating the home temperature as it lacks sensory control plus the expulsion of irritant gases for respiratory tract \(^{(14)}\). Furthermore, viral RTIs are frequently exacerbated by superimposed bacterial pathogens that may progress to LRTIs and pneumonia \(^{(15, 16)}\). Additional worsening factors include the presence of displaced and refugee peoples who complain about bad living conditions besides the general neglect of their health status. In this regard, the WHO since 2015 declared that there were 6.9 million Iraqis need immediate access to essential health services and 7.1 million need access to water, sanitation, and hygiene assistance \(^{(17)}\). Of note, a recent report found a significantly high infection rate of hMPV among homeless people \(^{(18)}\).

Actually, the results presented here are part of the larger studies including molecular detection of Human Respiratory Syncytial Virus (HRSV), Human Parainfluenza type 1 and 3 viruses (HPIVs) plus the HMPV which had the highest detection rate compared to other respiratory viruses. Furthermore, the HMPV positivity rate was significantly higher during season II compared to season I. It is definitely recognized that respiratory tract infections are one of the biggest health problems experienced by Iraqi health authorities, particularly among infants and children. However, limited studies were conducted in Iraq concerning the rate of HMPV infection, for instance, in Baghdad, two studies were reported controversial rates of hMPV (1.33% and 16%) respectively, while in Iraqi-Kurdistan, the rate of HMPV infection among children suffering from respiratory diseases and flu-like illness was high as compared with the results of current study (13.4% to 29.74%) \(^{(19, 20, 21, 22)}\).

Undoubtedly, Studies from around the world had yielded variable detection rates of HMPV, for example in Bangkok, Pakistan, Norway and Brazil, the HMPV was detected among hospitalized children with RTIs in (3.6%, 7%, 7.3% and 20%) respectively \(^{(23, 24, 25, 26)}\). In Saudi Arabia, Ali et al.,\(^{(27)}\), had reported a detection rate of HMPV similar to that obtained in the current study (9.9%), while in Egypt, El-Sawaf et al.,\(^{(28)}\), reported a higher detection rate of HMPV (16%). Certainly, The detection rate of HMPV is affected by several important factors such as seasons of infection, patients age, geographical region, methods of sampling, type of specimens, diagnostic methods, different viral strain and criteria of patients participating in the study from which the specimens were taken \(^{(29)}\).

The current study found that the detection rate of HMPV was significantly higher in season II (autumn period) with a peak in November and December compared to the season I (93.3% vs 6.7%, P= 0.0001). This seasonal pattern is not coinciding with data from other regions and countries. For instance, Atiyah et al.,\(^{(20)}\) found that the detection rate of HMPV was higher in October and November with a peak of cases in January. Furthermore, Hassan et al.,\(^{(22)}\), demonstrated that the majority of respiratory infections among children were recorded between November and March, with a peak in January and February, confirming that late autumn to early spring is the flourishing season for respiratory viruses in Kurdistan. In Iran, Moattar et al.,\(^{(30)}\) revealed that the rate of HMPV detection was increasing in winter and spring.

In Kuala Lumpur- Malaysia, a higher detection rate of HMPV was documented in March 2012, April and November 2013 \(^{(31)}\). Previous studies from Egypt, Kurdistan, and Pakistan, showed that the majority of hMPV infection was detected in the winter months with peak rates in February \(^{(32, 19, 33)}\). The high detection rates of HMPV during the second season may be partly due to the sharp change in climate and to the type of collected specimens.

Regarding the age at infection, previous serologic analyses have indicated that the majority of children less than 5 years in Europe and North America have been infected with HMPV, probably because they are highly susceptible to infections with this virus \(^{(34, 35, 4)}\). Additionally, the current results are consistent with most previous studies suggesting that the infection with hMPV was a highest among children <5 years old and...
decreased gradually with increased age, despite the fact that some of these studies were reported a few cases of hMPV infection in other age groups (36, 37, 20, 25, 26).

The symptoms of HMPV infection as a new pathogen of the most pediatrics viral respiratory infections, and those caused by other respiratory viruses are similar and include cough, fever, sore throat, runny nose, wheezing; bronchiolitis and pneumonia are the most frequent clinical manifestation associated with HMPV infections (18). In concurrence with the most other studies (38, 39, 7) the results of the current study showed that most patients with cough are the most infected with HMPV. In addition, the current results showed a high rate of HMPV-positive specimens was isolated from children with bronchiolitis; this finding is consistent with data from previous studies which showed that bronchiolitis was the most frequent clinical diagnosis among HMPV positive cases (40, 41, 42). In a related context, Williams et al., (36) reported that bronchiolitis and pneumonia are the most frequent discharge diagnoses were observed among HMPV positive children.

The study concluded that HMPV has an important role as a viral cause of the high rate of RTIs in the Diyala community, especially among children. The seasonal variation of HMPV can be observed in this study. Emerging HMPV strains are continually evolving.

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