

## MULTIPLEX SYBR GREEN ASSAY FOR CORONAVIRUS DETECTION USING FAST REAL-TIME RT-PCR

H. A. Salah<sup>1</sup>H. Y. Fadhil<sup>1\*</sup>F. G. Alhamdani<sup>2</sup>

Researcher

Assist. Prof.

Consult Doctor

<sup>1</sup>Dept. Biol. Coll. Sci. University of Baghdad.<sup>2</sup>Dept. Virol. National Central Public Health Lab. Ministry of Health.

\* Corresponding Author E-mail: hulayounis@yahoo.com

### ABSTRACT

This study was aimed to provide a local database for detection of coronavirus (CoV) species in suspect individual with respiratory tract infections like influenza type A and a tuberculosis using multiplex Sybr green reverse transcriptase real-time PCR (rRT-PCR) technique. A total of 500 samples was collected from individuals suffering from upper and/or lower respiratory tract diseases for testing of 4 CoV species (229E, OC43, NL63, and HKU1). RNA extracted, amplified and subsequent the positive samples sequencing. The results showed melting curve analysis (Tm) of the specific amplicons (79.73±0.36) and 9% positive for CoVs and some of them have other co-infection such as influenza virus 26.67%, and TB 11.11%. On the other hands, the CoVs were detected 4.62% in upper respiratory samples and 20.39% with lower respiratory samples. Sequencing results pointed out two isolates were CoV-NL63 and four isolates were CoV-229E, with first record accession number MN086823.1 and MN086824.1, respectively in GenBank. In conclusion, this rRT-PCR showed the rapid and efficient detection of CoVs with few copies number. This allows being used for the diagnosis of CoVs along with other respiratory viruses in a multiplex assay to reduce processing time. Subsequent applied nested RT-PCR to overcome the low viral load.

**Keywords:** Influenza type A, Tuberculosis, Iraqi provinces, URTI, LRTI.

صالح وآخرون

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الفحص المتعدد باستخدام صبغة السايبر الخضراء للكشف عن فيروسات الكورونا بتقنية تفاعل سلسلة البلمرة اللحظي (RT-PCR)

فيصل غازي الحمداني<sup>2</sup>حُلا يونس فاضل<sup>1\*</sup>هاله عبد الغفور صالح<sup>1</sup>

طبيب استشاري

أستاذ مساعد

طالبة ماجستير

<sup>1</sup>قسم علوم الحياة/كلية العلوم - جامعة بغداد. <sup>2</sup>قسم الفيروسات/مختبر الصحة المركزي - وزارة الصحة.

المستخلص

هدفت الدراسة إلى توفير قاعدة بيانات محلية للكشف عن أنواع فيروس الكورونا في الأشخاص المصابين بالتهابات الجهاز التنفسي مثل الأنفلونزا والسل باستخدام تقنية تفاعل سلسلة البلمرة العكسي اللحظي لصبغة السايبر الخضراء (rRT-PCR). جمعت 500 عينة من الأفراد الذين يعانون من أمراض الجهاز التنفسي العلوي و السفلي لاختبار أنواع الفيروس وهي 229E ، OC43 ، NL63 ، HKU1. استخلص الحمض النووي الريبي ، ضخمة وبعد ذلك اجري فحص تسلسل القواعد النيتروجينية للعينات الموجبة. أظهرت النتائج تحليل منحني الانصهار (Tm) لتضخيم المادة الوراثية الخاصة بالفيروس (79.73 ± 0.36) وكانت 9% إيجابية CoV وبعضها مصاب بعدوى مشتركة كفيروس الأنفلونزا 26.67% ، والسل 11.11% . من ناحية أخرى ، تم الكشف عن 4.62% CoVs في عينات الجهاز التنفسي العلوي و 20.39% في الجهاز التنفسي السفلي. أشارت النتائج اختبار تتابع القواعد النيتروجينية إلى عزلتين هي CoV-NL63 وأربعة عزلات كانت CoV-229E ، وسجلت لأول مرة في بنك الجينات العالمي MN086823.1 و MN086824.1، على التوالي. استنتجت الدراسة ، بأن اختبار rRT-PCR هذا هو كشف سريع وفعال بالكشف عن أنواع CoV ذو محتوى فيروسي قليل بالعينة. هذا يسمح باستخدامه لتشخيص الاصابات المصاحبة للفيروسات جنبا إلى جنب مع فيروسات الجهاز التنفسي الأخرى في اختبار متعدد لتقليل وقت التشخيص. تطبيق تقنية تفاعل سلسلة البلمرة العكسية المتداخلة RT-PCR للتغلب على انخفاض الحمل الفيروسي.

الكلمات المفتاحية: فيروس الأنفلونزا، مرض السل، المحافظات العراقية، الاصابات التنفسية العلوية، الاصابات التنفسية السفلية.

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## INTRODUCTION

Coronaviruses (CoVs) belonging to the family *Coronaviridae* in the order *Nidovirales* that infect the respiratory tract, a group of large enveloped RNA viruses between 70 and 120 nm in size, with a helical nucleocapsid and carries extended spike proteins on the membrane surface, providing the typical crown-like structure (crown = corona) seen by electron microscopy, positive single-stranded with the largest genome of all known RNA viruses (27 to 33 kb) (30). In addition, there is a high nucleotide substitution rate across the genome (11). The genetic recombination of coronaviruses had possibly led to the emergence of lethal pathogens such as SARS-CoV and MERS-CoV, which caused up to 50 % mortality in infected individuals. Both SARS-CoV and MERS-CoV is phylogenetically closely related to coronaviruses in bats (17). Coronaviruses could cause both human and veterinary outbreaks owing to their ability to recombine, mutate, and infect multiple species and cell types, so they have the propensity to jump between species. But by now, there is no anti-viral therapeutics that specifically target human coronaviruses, and only limited options are available to prevent coronavirus infections (4, 16). The Alpha-CoV genus includes human CoV HCoV-229E and HCoV-NL63, and other members of the genus Alpha are porcine epidemic diarrhea virus (PEDV) and some bat CoV. The Beta-CoV genus comprises than Alpha-CoV and contains four distinct lineages: A (HCoV-HKU1), B (SARS-CoV), C (MERS-CoV), and D (HCoV-OC43) (21). Sybr green reverse transcriptase real-time PCR (rRT-PCR) analysis for detecting HCoVs from acute respiratory tract infections because it has many benefits. This test was simple to use, rapid, sensitive and accurate detection of HCoVs, cost and time-effective, so it regarded an alternative strategy for high-performance screening of suspected samples from humans, animals and environments compared to the TaqMan probe-based assays, Sybr green-

based assay was slightly more sensitive. (10, 13), especially for primer design and optimization procedures (5). Thus, this study aimed to provide a local database for detection of CoV species in suspect individual with respiratory tract infections like influenza type A and a tuberculosis using multiplex Sybr green rRT-PCR technique.

## MATERIALS AND METHODS

### Study population

A total of 500 respiratory samples was collected from individuals between November 2018 to April 2019 at different Iraqi provinces. The specimens included upper respiratory 348 (swabs), and lower respiratory 152 (tracheal aspirates). The samples had been collected for routine viral diagnostic screening of people suffering from upper and/or lower respiratory tract diseases for testing of coronaviruses species (229E, OC43, NL63, and HKU1).

### RNA extraction

All upper respiratory tract samples (URT) (348 swabs distributed 272 that gave negative and 76 positive results of influenza test) and also all lower respiratory tract samples (152 lung washing included 17 sample positive for TB) were selected for RNA extraction to detect five human coronaviruses by using a specific kits: QIAamp Viral RNA Mini Kit (GmbH, Hilden, Germany) as described according to manufacturer's instructions. RNA extraction stored at deep freeze -80 C° until the use (2).

### Fast Real-Time Reverse transcription-Polymerase Chain Reaction (rRT-PCR) with Sybr green

All the clinical samples were tested by rRT-PCR multiplex for detecting four CoV species (229E, NL63, OC43 and HKU1) with specific primers using Sybr green. Four pairs of specific primers were used to rRT-PCR detection and amplify the RNA-dependent RNA polymerase (**RdRp**) gene of HCoVs (229E, HKU1, OC43 and NL63) which contains ORF1a and ORF1b as mentioned in table 1 for all upper and lower respiratory samples (8).

**Table 1. Sequences of primers using in Fast rRT-PCR**

Virus	Primer	Sequence 5'-3'
HCoV 229E	Forward	CAGTCAAATGGGCTGATGCA
	Reverse	AAAGGGCTATAAAGAGAATAAGGTATTCT
HCoV OC43	Forward	CGATGAGGCTATTCCGACTAGGT
	Reverse	CCTTCCTGAGCCTTCAATATAGTAACC
HCoV NL63	Forward	GACCAAAGCACTGAATAACATTTTCC
	Reverse	ACCTAATAAGCCTCTTTCTCAACCC
HCoV HKU1	Forward	CCTTGCGAATGAATGTGCT
	Reverse	TTGCATCACCCTGCTAGTACCAC

In Sybr green detection, the master mix for the one step RT-qPCR (Promega) (1) was prepared. The PCR tube 5 µl of the GoTaq qpcr Master Mix; 2x, 0.15 µl of each primer for HCOV-HKU1, HCOV-NL63 and HCOV-OC43, 0.2 µl of each primer for HCOV-229E and 0.5 µl of enzyme mix and mixing gently by pipetting then distributed 6.5 µl of the master mix to rRT-PCR wells and added 3.5 µl of the RNA template. The plate was transferred to Applied Biosystem Fast rRT-PCR 7500 apparatus (28). Following amplification, a melt curve analysis was performed to verify the specificity of the amplified products by their specific melting temperatures (T<sub>m</sub>). The amplification was performed RT step activation at 50°C for 30min, initial denaturation at 95°C for 15min, followed 45 cycles: 95°C for 3sec then 55°C for 4 sec, and hold stage at 72°C for 30sec (5).

#### Detection by conventional RT- PCR

All the positive samples in fast rRT-PCR for four HCoVs species were chosen to implement the conventional RT-PCR and subsequences tested for sequencing. One pair of specific primers was used in the amplification process of the conserved region of Human coronaviruses, spike (S), nucleocapsid (N) proteins and RNA dependent RNA polymerase (polymerase coding region-1b). Forward hcov: (5'-ATG GGW TGG GAY TAY CCH AAR TGT G-3'), Reverse -1 hcov: (5'-CCW CAY GAR TTY TGY TCM CAR CA-3') and with the product size of about 600 base pairs used for detection and sequencing of the designated virus (26). Superscript TM III Platinum TM One-step RT-PCR kit (Invitrogen, USA) was performed to prepare solution for amplification of RNA templates by conventional RT-PCR (12). A master mix tube contains all the components included 12.5 µl reaction buffer (5x), 0.5 µl Super Script TM III RT/Platinum TM Taq mix, 0.8 µl of each

primer, and then added to 10 µl RNA template in 25 µl final reaction. After optimization, the amplification program was 50 °C for 30 min and 95 °C for 2min RT activation and denaturation, following cycling stage 35 cycle: 94 °C for 30sec, 56 °C for 30sec and 72 °C for 1min. The final extension was 72 °C for 7min.

#### Gel electrophoresis

Amplified RT-PCR products were electrophoresed through 1.5% agarose gel in TBE buffer for 1hr at 80 volt. Target bands of specific primers were visualized by staining with ethidium bromide (4%) dye solution along with ladder bands (100 pb). Positive and negative controls were included in each run. The results were counted as valid when there is no false-positive result was observed in the negative control reactions and when the positive control sample was positive, visualized under UV light at 350 nm (23).

#### Sequencing for conventional RT-PCR product

The gene sequencing process was conducted on human coronaviruses in this study, the conventional RT-PCR product was sent to Macrogen Company, USA by using forward primer for the polymerase coding region (1b) gene on genetic analyzer (Applied Biosystems). Homology search was performed using Basic Local Alignment Search Tool (BLAST) program online using blastn and blastx algorithms which are available at the National Center Biotechnology Information (NCBI) online at (<http://www.ncbi.nlm.nih.gov>). Bioedit program (v7.2) used for multiple sequence alignment in ClustalW and to calculate the similarity matrix between the viral strains. To classify the HCoV species, phylogenetic tree was performed applying the neighbor-joining method and the genetic distances were calculated according to the maximum composite likelihood model with 1000-

bootstrap using the MEGA version 6.0 software. A bootstrap value of 70 was used as an indicator of the significance of the clusters. The results were compared with the GenBank database published in ExPASy program for amino acid sequence and open reading frame, then compared with Blastx in GenBank; the coding DNA sequence (CDS) regions detected in Blastn and compared with open reading frames (ORF- Finder). FinchTV program was applied to determine the quality of the nucleotide sequence analysis. After the sequencing run, two local samples sequencing of nucleotides submitted to GenBank and reported with accession numbers.

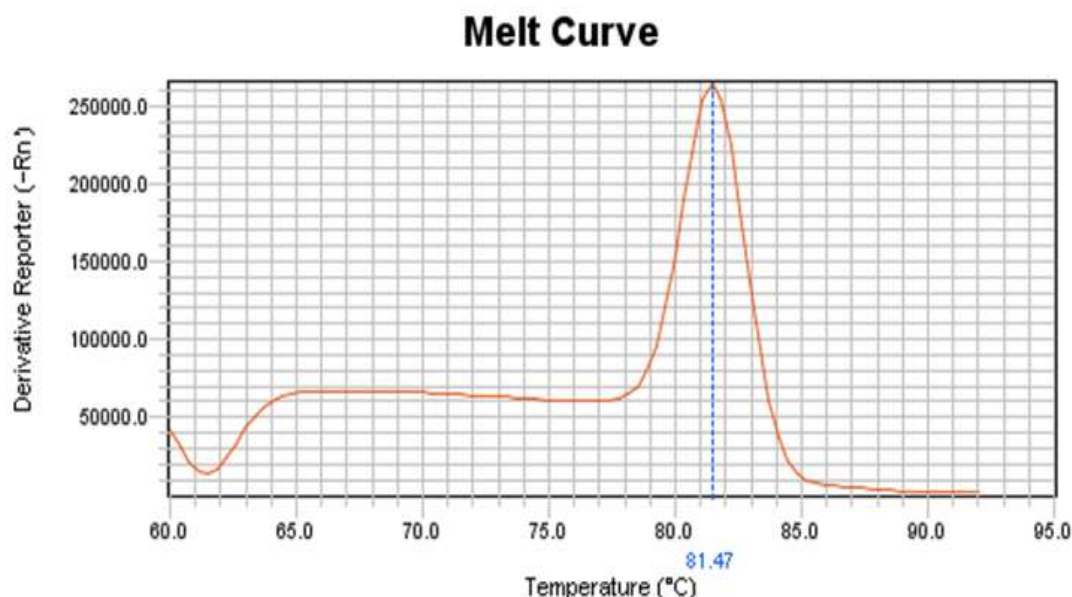
### Statistical analysis

The statistical analysis system was analyzed by IBM SPSS Statistics version 25. All values, proportions and their frequencies were checked by applying the Pearson chi – square (X<sup>2</sup>) and cross tab test to investigate significant comparison between viral infection percentages in different studying markers of population study. Also, the odds ratio and fisher exact test for the appearance of HCoV infections along with influenza type A and tuberculosis (TB) infections were calculated for cohort human coronavirus patients. A

value of  $P < 0.05$  was considered statistically significant.

### RESULTS AND DISCUSSION

The positive results were found in Sybr green rRT-PCR on the basis of their melting curve analysis that revealed more than 70 with a mean melting temperature ( $T_m$ ) of the specific amplicons was  $79.73 \pm 0.36$  (mean  $\pm$  SE). The analysis of result of amplification plot is studied in conjunction with melting curve (10). The  $T_m$  was the most important factor of PCR products, which must be distinct in each primer pair for the specific identification of each virus, according to the melting curve (28). Finding the  $T_m$  of HCoV positive samples was (81.47) with high in the number of viruses (Fig. 1) like in a sputum sample with cycle number (Ct) 24, whereas there is low and high in viral load in other cases with the peak of  $T_m$  analysis was decreased to 73.67. This indicates that peak of melting curve analysis effected with viral load (Ct value) and viral species in different respiratory tract samples. Other investigators have shown the four HCoVs can be distinguished from each other by specific melting peaks, but there is small difference in  $T_m$  values between amplicons of HCoVs species may result in weak discrimination (29).



**Figure 1. Melting curve of the amplification HCoV positive sample with high viral load (Ct 24) and ( $T_m$  81.47) detection based on Sybr green rRT-PCR assay for sputum samples of a patient with pneumonia**

Overall, the result showed 45/500 (9%) positive for HCoVs of patients have respiratory tract infections and some of them

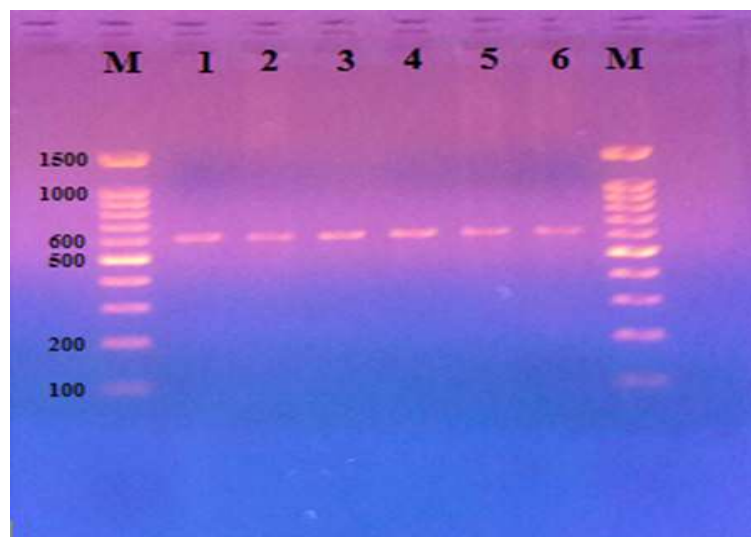
have other co-infection such as influenza virus, TB and pneumonia or were immunocompromised peoples. On the other

hands, the HCoV were detected 14/348 (4.62%) positive of the total upper respiratory samples and 31/152 (20.39%) positive of the total lower respiratory samples due to most of the patients were suffering acute respiratory tract infections during influenza season. The negative remaining samples of the total upper and lower respiratory samples may be patients have the same symptoms, but strongly associated with other viruses, bacteria and fungi, for instance the respiratory syncytial virus (RSV) (14, 15), influenza A and B; parainfluenza 1, 2, and 3 (25); adenovirus (19), human metapneumovirus (Hmpv) (6) and bocavirus (7). Greenberg (18), observed the four species of HCoV OC43, 229E, NL63, and HKU1 are associated with a wide range of URTI and occasionally in LRTI, including pneumonia and bronchitis. In contrast to our study, which showing, positive cases of HCoVs by using rRT-PCR were recorded in LRT more than in URT, with rates 31/45 (68.9%) and 14/45 (31.1%) respectively. This may belong to the LRT samples collected from the patients more infected of severe respiratory tract infections such as TB, bronchitis and pneumonia with than other patients less infected. So, these severe diseases may cause decreased in the immunity system status of the patients and makes them more susceptible to infect with circulating HCoVs. Also, most of the LRT samples collected from hospitalized patients. Although the four HCoVs are distributed globally with various prevalence rates (1.6%–16%) in different countries/regions (27, 22, 31), but no previous study was done to detected circulating HCoVs in Iraq yet, this is the first record and considered the key reference for further studies over it. It is interesting to note, the results pointed out 12/45 (26.67%) co-infected with the most frequently Flu A and the relative risk value represented 21.5 (95% CI 4.9-93.9) of cohort influenza type A positive case. Moreover, the co-infection with pulmonary tuberculosis was 5/45 (11.11%) of HCoV cases and the relative risk value 1.53 (95% CI 0.68-3.45) of cohort TB positive cases. Coronaviruses are frequently co-detected with

other respiratory viruses, particularly with the influenza type A and human respiratory syncytial virus (HRSV) in upper respiratory infections (20, 33). While the bacterial infections in lower respiratory tract like pulmonary tuberculosis, which decreasing in the immune system status, so do the patient more susceptible for infecting with more than circulating HCoVs and may be due to interaction between various HCoV species themselves (3). Moreover, there is no previous study documented for HCoV appearance with TB infections. The most important observation in this study, we found some patients infected with HCoV, who were co-infected with pulmonary TB. This may be a causative agent for increasing HCoVs detection in LRT than in URT. Co- infections are commonly related and found mostly among patients with high viral load than those with low viral load indicating that patients with high HCoV viral load were more likely to be co-infected with other infectious diseases such as TB, hence HCoV co-infection with TB appearance may increase with the severity of pulmonary disease.

#### **Conventional RT-PCR Amplification**

All positive samples (45) in the rRT- PCR assay were subjected to conventional RT-PCR amplification using polymerase coding region (b1) gene primers. The Results showed 6/25 (24%) have high viral load of positive samples which appeared Ct <20 (cycle number) of the amplification curve in the rRT-PCR, appeared peak in the curve when detected by Sybr green-melt curve rRT-PCR and observing the specific band with RT-PCR assay when an RNA product runs in 1.5% agarose gel in the gel electrophoresis and specific band (Fig. 2). But the remaining HCoV positive samples failed in observing the specific band with RT-PCR assay because of these samples were low viral load with Ct more than 25 in rRT-PCR. As well as this may belong to the high sensitivity of rRT-PCR other than RT-PCR (24, 32). HCoVs specific amplicons in this assay had unique Tm values, significantly different from those of primer dimers to avoid false positive results (13).



**Figure 2. The amplification of a polymerase coding region (1b) gene of HCoVs positive samples in lane 1-6 by using RT- PCR (lane 1 and 4 are NL63; lane 2, 3, 5 and 6 are 229E). Amplicons size was about 600 bp. The amplicons were run on agarose gel 1.5% and visualized with Transilluminator, M: Marker (ladder) ranged 10000-100 bp**

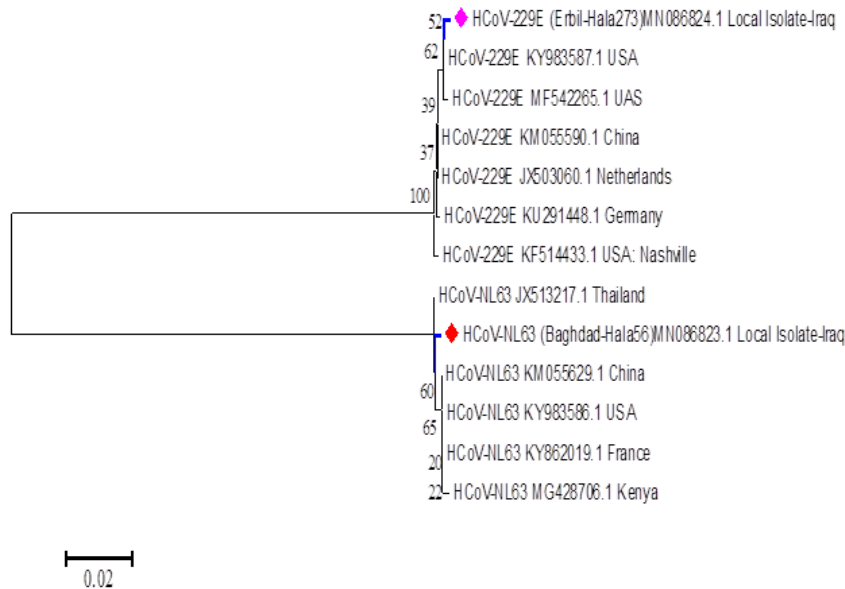
#### **HCoV polymerase coding region (1b) gene sequence blast of local isolates with database of NCBI**

To understand the genetic variation and characterization of HCoVs in the current study, six positive samples were selected RT-PCR amplification and sequencing the polymerase coding region (1b) gene. Nucleic acid sequencing was conducted on RT-PCR products to emphasize their specificity and introduce the ultimate means to identify the viral genome and detect the virus. In this study; amplicons have HCoV isolated sequence of conserved regions that had not been previously sequencing recorded in Iraq. Consequently, six local HCoV isolates were analyzed and compared with a reference strain available in the GenBank database NCBI. After using the BLAST program which is available at the NCBI, the result of sequencing appeared 99% compatibility with reference human coronavirus polymerase coding region (b1) gene and alignment with references HCoVs from NCBI. The bit score is defined as a statistical measure of the moral similarity and the higher value indicated that the high degree of similarity, and if dropped from the class of 50 points, the sense that there is no similarity. Expected value (E) is defined to give an estimate of the number of times expected to get the same similarity coincidental and lower the E-value. This indicates that the degree of similarity was high between sequences which give greater

confidence. The value of a very close to zero means that these sequences are identical (9).

#### **Sequence similarity matrix and phylogenetic tree of HCoV isolates (229E and NL63-HCoV) for RdRp gene**

This is the first molecular report on the finding of HCoV isolates which one of the causes respiratory tract infections in all age in Iraq. Sequence similarity values were calculated for sequencing nucleotides using BioEdit program. Local Isolate Iraq (HCoV-NL63 Baghdad-Iraq MN086823.1) showed greatest sequence similarity (99%) to HCoV-NL63 JX513217.1 Thailand, HCoV-NL63 KY983586.1 USA, HCoV-NL63 KY862019.1 France and HCoV-NL63 MG428706.1 Kenya. Additionally, the sequence similarity matrix showed 99% similarity between the other Local Isolate Iraq (HCoV-229E Erbil-Iraq MN086824.1) which were HCoV-229E KY983587.1 USA, HCoV-229E KM055590.1 China, HCoV-229E MF542265.1 UAS, HCoV-229E JX503060.1 Netherlands, HCoV-229E KU291448.1 Germany and HCoV-229E KF514433.1 USA: Nashville. In the phylogenetic tree, we can see the Iraqi\local isolates (HCoV-229E and –HCoV-NL63) (Fig. 3), which the first recorded by local isolates of HCoV species in GenBank, were divided into two subgroups. HCoV-229E being more similar to strains from the USA with the same subgroup, while HCoV-NL63 related to USA, France and Kenya strains in another subgroup.



**Figure 3. Phylogenetic tree analysis of nucleotide sequences of RdRp gene of Human coronavirus isolates detected in Iraq. The Iraqi isolate was indicated by the symbol (◆). The pink symbol color was indicated on 229E-HCoV isolate of Erbil (MN086824). The red symbol color was indicated on NL63-HCoV isolate of Baghdad (MN086823). The evolutionary history was inferred using the Neighbor-Joining method. The phylogenetic tree was conducted in MEGA6**

The assessment of this research gives extra evidence for the concept that HCoV species is available worldwide, due to the similarities among our local isolations with the international, incidence may differ substantially depending on the species with particular locations. In conclusion, the rRT-PCR based on Sybr green showed the rapid and efficient detection of CoVs with few copies number. This allows to be used for the diagnosis of CoVs along with other respiratory viruses in a multiplex assay to reduce processing time. Subsequent applied nested RT-PCR to overcome the low viral load.

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