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Nucleocapsid gene analysis from an imported case of Middle East respiratory syndrome coronavirus, Malaysia

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ABSTRACT

Objective: To describe the complete nucleocapsid (*N*) gene region of Middle East respiratory syndrome coronavirus (MERS-CoV) from imported case in Malaysia and the relations with human- and camel-derived MERS-CoV.
Methods: Combination of throat and nasal swab specimens was subjected to viral RNA extraction. For screening, the extracted RNA was subjected to real-time RT-PCR targeting upstream of *E* gene, open reading frame 1b and open reading frame 1a. For confirmation, the RNA was subjected to RT-PCR targeting partial part of the RNA-dependent RNA polymerase and nucleocapsid, followed by amplification of complete *N* gene region. Nucleotide sequencing of the first Malaysian case of MERS-CoV was performed following the confirmation with real-time RT-PCR detection.
Results: Initial analysis of partial RNA-dependent RNA polymerase and *N* gene revealed that the nucleotides had high similarity to Jeddah_1_2013 strain. Analysis of complete *N* gene region (1 242 nucleotides) from the case showed high similarity and yet distinct to the nucleotide sequences of camel-derived MERS-CoV.
Conclusions: From the finding, there are possibilities that the patient acquired the infection from zoonotic transmission from dromedary camels.

1. Introduction

Middle East respiratory syndrome coronavirus (MERS-CoV) is a new coronavirus which belongs to lineage C of the genus *Betacoronavirus* and was first isolated from a patient with severe respiratory disease and renal failure in 2012. As of 13 June 2014, a total of 697 laboratory-confirmed cases with 210 deaths have been reported worldwide[1]. All these cases were reported among people either from or have travelled from the Arab Peninsular.

Most people infected with MERS-CoV and developed severe respiratory illnesses with symptoms of fever, cough, and shortness of breath. About 30% of the cases have succumbed to their illness and majority of those who died had underlying medical condition such as diabetes, cancer, and chronic lung, heart and kidney disease[2].

On 15 April 2014, Malaysia has confirmed the first case of MERS-CoV infection in a 54 year-old man, who came back from Saudi Arabia after performing the umrah. He was a known diabetic and was on medical treatment. The patient had departed from Jeddah and arrived in Malaysia on 29 March 2014; and on 10 April 2014, he was admitted to one of the local hospitals for treatment of severe acute respiratory infection. However, he succumbed to his illness on 13 April 2014[3].

Combination of throat and nasal swab specimens taken from the patient was sent to a state hospital laboratory that has the capability to perform real-time RT-PCR (rRT-PCR) assay for MERS-

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CoV. An rRT-PCR screening assay targeting upstream of *E* gene (upE) and an rRT-PCR confirmatory assay targeting open reading frame (ORF) 1a gene were performed at the state hospital and both assays were positive for MERS-CoV[3]. Since this is the first case that has ever yielded a positive result in Malaysia, the sample was sent to the Virology Unit, Institute for Medical Research, Kuala Lumpur which acts as the national MERS-CoV confirmation laboratory, for further investigations. In this study, the complete *N* gene region of MERS-CoV of the first Malaysian case was analyzed and it was compared genetically with other human cases and virus identified in camels.

2. Materials and methods

2.1. Nucleic acid extraction and PCR

Combination of throat and nasal swab specimens received by Institute for Medical Research on 15 April 2014 was subjected to viral RNA extraction by using QIAamp Viral RNA Mini Kit (Qiagen, Germany) following the manufacturer's instruction.

For screening, the extracted RNA was subjected to rRT-PCR targeting upE, ORF1b and ORF1a[4,5], which were optimized for SuperScript III Platinum One-Step RT-PCR System with Platinum Taq Polymerase (Invitrogen, USA). For confirmation, the RNA was subjected to RT-PCR targeting partial part of the RNA-dependent RNA polymerase (RdRp) and nucleocapsid (N)[5], followed by amplification of complete *N* gene region designed for subsequent phylogenetic analysis (Table 1).

Table 1

Primers used for amplification and sequencing of the Malaysian case.

Primer names and sequences	Position*
For amplification and sequencing	
NF1: 5'-ATGGCATCCCCTGCTGCACCTCGT-3'	28561-28373
NR1: 5'-CATCTTATTTTTAGCAGCAGCAGCATCT-3'	29310-29283
For sequencing	
NF2: 5'-AGCCAAAAGTAATCACTAAGAAAGATGCT-3'	29261-29289
NR2: 5'-CTAATCAGTGTTAACATCAATCATTGGA-3'	29591-29564
N350R: 5'-TTAACAGCCCGGAATGGGAGTGCT-3'	28887-28910

F: forward; R: reverse.

*: Referring to the sequence of MERS-CoV strain Jeddah_1_2013 with GenBank accession No: KJ556336.

For the amplification of partial *RdRp* gene region, primer RdRpSeq-Fwd and RdRpSeq-Rev were employed[5], while primer NSeq-Fwd and NSeq-Rev were used for amplification of partial *N* gene region[5]. The RT-PCR amplifications were performed by using MyTaq™ One-Step RT-PCR Kit (Bioline USA), following the parameters: 45 °C for 20 min, polymerase activation at 95 °C for 1 min followed by 45 cycles at 95 °C for 15 seconds, 56 °C for 15 seconds and 72 °C for 30 seconds and terminal elongation at 72 °C for 2 min. For amplification of RdRp, second-round PCR reaction was performed with RdRpSeq-Fwd (similar to the first round) and RdRpSeq-Rnest primer by using MyFi™ Mix Kit (Bioline, USA) according to the following parameters: 95 °C for 1 min, followed by 35 cycles at 95 °C for 15 seconds, 56 °C for 15 seconds, 72 °C for 20 seconds, and 2 min at 72 °C for terminal elongation[5]. The resulted amplicons were separated by gel electrophoresis, and the DNA fragments were excised, purified and subjected to sequencing in both directions. The obtained sequences were verified by basic local alignment search tool.

As for the amplification of complete *N* gene region, cDNA was synthesized by using SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen, USA) with NR2 primer (Table 1). Two microliters of the generated cDNA was subjected to one round of PCR cycle by using MyFi™ Mix Kit (Bioline, USA) with NF1 and NR2 (Table 1) primers following the parameters: 95 °C for 1 min followed by 25 cycles at 95 °C for 15 seconds, 52 °C for 15 seconds and 72 °C for 30 seconds and terminal elongation at 72 °C for 5 min. Then, 0.5 μL of the resulted PCR amplicon was further subjected to the second round PCR following the above-mentioned parameters. The resulted PCR product was separated by gel electrophoresis while the DNA fragment was excised, purified and subjected to sequencing by using NF1, NR1, NF2, NR2 and N350R primers.

2.2. Phylogenetic analysis

The obtained sequences were analyzed by using ChromasPro version 1.7.4 (Technelysium Pty Ltd, Australia). Multiple sequence alignment was performed by using Clustal X (version 2.0.11) with 44 human-derived (including the Malaysian case; MyH010414; GenBank accession No: KP769415) and 3 camel-derived MERS-CoV sequences[6]. Maximum likelihood analysis was conducted by using Molecular Evolutionary Genetics Analysis 6.06[7]. The evolutionary history was computed by using Tamura-Nei model with 1 000 replicates of bootstrap analysis.

3. Results

Extracted RNA from combination of throat and nasal swabs from the case was detected positive both in rRT-PCR and conventional RT-PCR for partial *RdRp* and *N* gene. The cycle threshold values for upE, ORF1a and ORF1b were 28.19, 29.16 and 33.36, respectively. Amplification of partial *RdRp* and *N* gene regions resulted in DNA fragment of ~240 bp and ~310 bp, respectively (data not shown). Initial sequencing result of these fragments showed highly identical nucleotide sequence (99%) to MERS-CoV strain Jeddah_1_2013 (KJ556336.1), further confirming the MERS-CoV infection.

Nucleotide analysis of the complete *N* gene region showed that the Malaysian case virus strain MyH010414 (GenBank Accession No: KP769415) was 99.9% identical to the sequence derived from the Qatari and Saudi Arabian camels (KJ650098, KJ713296 and KJ713295) with nucleotide difference only in 1 of 1 242 nucleotides. It is also noted that this strain showed 99.4% similarity to the first MERS-CoV case: HCoV_EMC/2012 differed by 7 of 1 242 nucleotides.

Phylogenetic analysis of MyH010414 sequence together with 43 human-derived MERS-CoV from 2012 to 2014 and 3 camel-derived MERS-CoV sequences are shown in Figure 1. Maximum-likelihood analysis of *N* gene region was constructed by using the Tamura-Nei model. Each branch showed, in Figure 1, the GenBank accession number followed by description of the sequence used.

It is noted that the nucleotide sequence from the Malaysian case is clustered together with human-derived MERS-CoV sequences isolated from human cases in 2014, among them are those reported in Greece and USA. The sequences of the camel-derived MERS-CoV group together in their own cluster, however, close to those of human-derived sequences isolated in 2014.

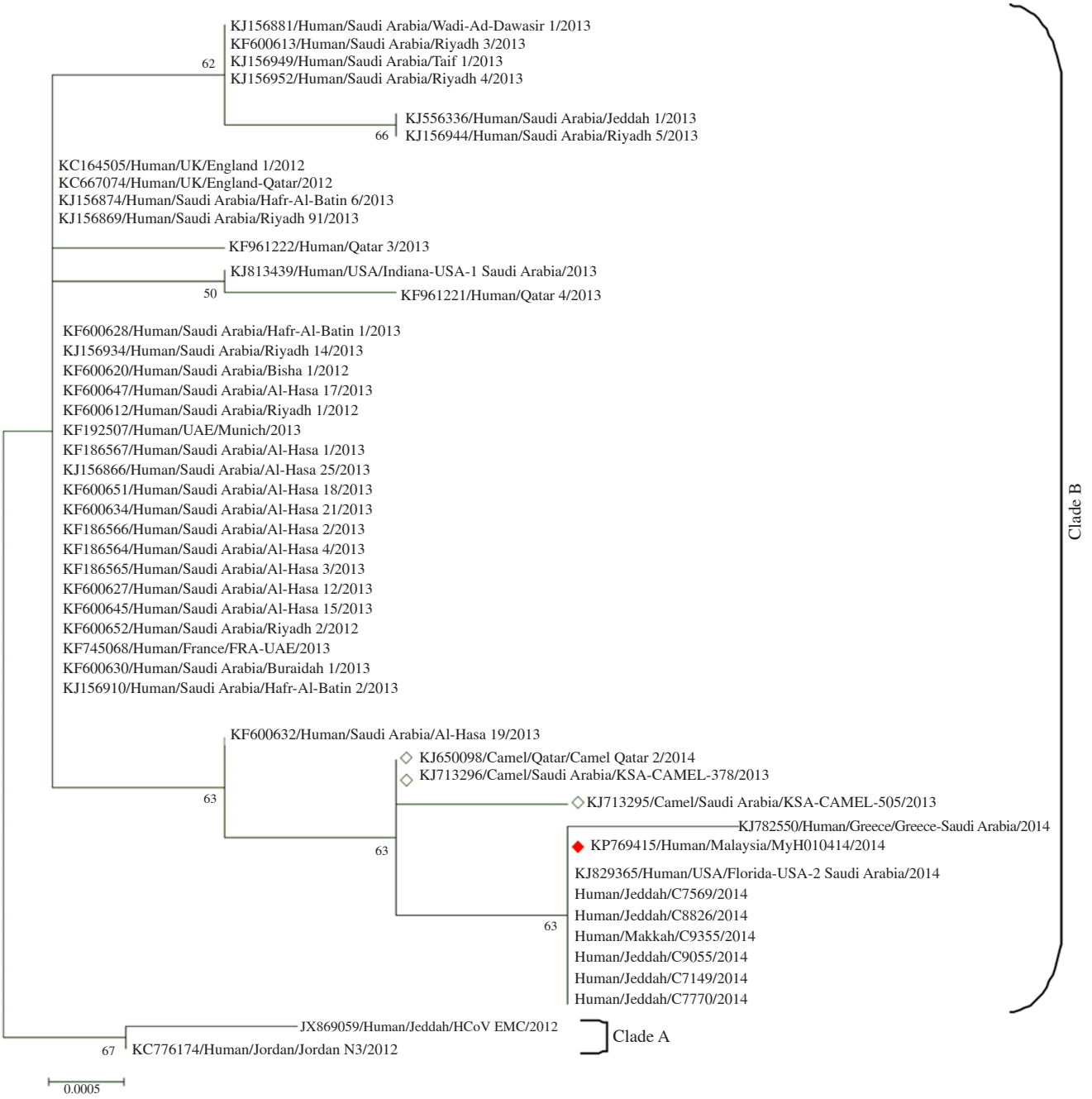

Figure 1. Phylogenetic analysis of *N* gene region of Malaysia MERS-CoV case together with 3 camel-derived and 43 human-derived MERS-CoV sequences from 2012-2014.

Red diamond: Malaysian case; Blank diamond: sequences from the camel-derived MERS-CoV.

4. Discussion

The sequence analysis of *N* gene region from the first case of MERS-CoV in Malaysia was reported. Comparative analysis of the *N* gene region from the Malaysian case and camel-derived MERS-CoV sequence showed more than 99% identities. The finding is concerted with results of studies performed in dromedary camel from Qatar[8], Saudi Arabia[9,10] and Oman[11], which showed that the camel-derived and human-derived MERS-CoV were highly similar yet distinct. This is also reflected in the phylogenetic analysis; the camel-derived and human-derived sequences were independently clustered.

Based on the travel history of the Malaysian case, the infection may be acquired after being exposed to infected camels, via patting the camel especially their nostril region and consuming the raw, unpasteurized camel milk[3]. Similar observation as the Malaysian case has been reported by Memish *et al.*[9], which substantiate the cross-species transmission to humans, with the possibility of dromedary camels being the primary reservoir hosts of MERS-CoV[8-11]. Juvenile camels may play a big role in the viral transmission, since the younger were shown to shed more virus compared to the old ones[9,10,12]. The transmission from camels to human was postulated to occur through very close contact with high infectious dose of virus via nasal route[11]. Other possible route of transmission is via consumption of raw milk from infected camels, which was supported by a report showing that MERS-CoV could survive slightly longer in raw camel milk in comparison to other species[13].

MERS-CoV in human emerged only recently, although it has been shown to be circulating in camels since 1992 or even earlier[10]. Even though higher frequency of MERS-CoV infection was observed in the camels of the Arabian Peninsula, low number of infections was recorded in human. Thus, further study to determine the exact mode of transmission from camel-to-human and human-to-human is important to mitigate the spread of this virus.

Conflict of interest statement

We declare that we have no conflict of interest.

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