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PII: S1201-9712(15)00228-3
DOI: http://dx.doi.org/doi:10.1016/j.ijid.2015.09.018
Reference: IJID 2440

To appear in: *International Journal of Infectious Diseases*

Received date: 31-7-2015
Revised date: 21-9-2015
Accepted date: 22-9-2015

Please cite this article as: Furuse Y, Okamoto M, Oshitani H, Conservation of nucleotide sequences for molecular diagnosis of Middle East respiratory syndrome (MERS) coronavirus, 2015, *International Journal of Infectious Diseases* (2015), http://dx.doi.org/10.1016/j.ijid.2015.09.018

Title

Conservation of nucleotide sequences for molecular diagnosis of Middle East respiratory syndrome (MERS) coronavirus, 2015

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Abstract

Because of the widespread infections due to Middle East respiratory syndrome coronavirus (MERS-CoV), we assessed protocols for molecular diagnosis by analyzing nucleotide sequences of the viruses detected between 2012 and 2015 including sequence from the big outbreak in eastern Asia in 2015. Although it has only been 2 years after the establishment of diagnostic protocols,

mismatches between the sequences of primers/probes and viruses were found for several assays. Such mismatches could lead to a lower sensitivity of the assay, further leading to false-negative diagnosis. Furthermore, we suggested a slight modification in the primer design. Protocols for molecular diagnosis of viral infections should be reviewed regularly after establishment, particularly for viruses that pose a great threat to public health, such as the MERS-CoV.

Keywords

Middle East respiratory syndrome coronavirus; diagnosis; laboratory; genome; sequence; evolution

Introduction

Middle East respiratory syndrome coronavirus (MERS-CoV) is an enveloped virus with a positive-sense RNA genome. Infection with the virus causes severe respiratory symptoms in humans with a case fatality rate as high as 37% [1]. Camels may be a source of infection to humans [2]. Human-to-human transmission is also possible but requires close contact such as health care related contact without proper measures for infection control and prevention [3]. The earliest case of MERS was first reported in Jordan; subsequently, in a short time, MERS-CoV was isolated from cases in Saudi Arabia [4]. Thereafter, infections have been endemic mainly in Middle East. Furthermore, MERS-CoV has spread sporadically to other areas such as Europe, North America, Africa, and Southeast and East Asia by travelers from the Middle East [5].

Laboratory diagnosis of MERS-CoV infection is mainly performed using real-time reverse transcription polymerase chain reaction (RT-PCR) to detect viral RNA in specimens. Interim recommendations by the World Health Organization (WHO) in 2015 for laboratory testing of MERS-CoV included protocols for RT-PCR that were developed by the University Hospital Bonn and the United States Centers for Disease Control and Prevention [6-9]. The document included 7 assays: 1) upE assay is considered highly sensitive and is recommended for screening [7], 2) ORF1a assay is considered equally sensitive as the upE assay [6], 3) ORF1b assay is considered less sensitive than the ORF 1a assay [6, 7], 4) N2 and 5) N3 assays can complement upE and ORF1a assays for screening and confirmation [8, 9]. To date, these assays have shown no cross reactivity with other

human coronaviruses [6-8]. Sequencing protocols for further confirmation, namely 6) RdRpSeq and 7) NSeq assays, were also developed [6].

Because MERS-CoV is an RNA virus, which can evolve rapidly, there is always a concern that these protocols are not suitable for detecting the current MERS-CoV because of the mismatch among sequences in the primer/probe regions. Here we analyzed recent viral genomic nucleic acid sequences to discuss the efficacy of the RT-PCR protocols for molecular diagnosis of MERS-CoV infections.

Findings and discussion

As of July 29, 2015, there were 386 sequence data for MERS-CoV at 'Virus Variation' (http://www.ncbi.nlm.nih.gov/genome/viruses/variation, GenBank accession numbers of analyzed sequence data are available upon request). The data included viruses detected between 2012 and 2015, including viral sequence from the big outbreak in eastern Asia in 2015 [1]. We obtained and analyzed these data on the 386 sequences, including complete as well as partial genome sequences. Sequence data were aligned by ClustalW to assess genetic changes in nucleotide sequences of the primer and probe regions of the assays described above. Number of viral sequences which match primer/ probe sequence perfectly were counted.

As mentioned previously in this report, UpE, ORF1a, N2, and N3 assays can be used for screening because of their high sensitivity [6-9]. Among these, only the primer and probe designs of

ORF1a assay showed 100% conservation of all sequence data available today (Table). We found minor mismatches for the UpE (1 nucleotide substitution in 2 sequences) and N2 (1 nucleotide substitution in 1 sequence) assays and significant mismatches for the N3 assay. The primer/probe regions are conserved well except for the N3 assay. Besides, mismatches were not found in 3' end region of primers for the UpE and N2 assays (Table). Sensitivity of the assays may not be affected a lot. No mismatches were found for ORF1b assay.

As for the sequencing assays, we found no sequence data that matched the sequence of the reverse primer for the RdRpSeq assay. However, we found a single common mismatch in all sequence data. When the mismatched nucleotide was corrected, the RdRpSeq assay matched all the sequence data perfectly (*corrected reverse primer*, Table). In addition, viral sequences of the reverse primer region for the NSeq assay were not highly conserved; the sequence matched only 49% strains. Based on these results, we here suggest a modified reverse primer for the assay to reduce the possibility of mismatch (*modified reverse primer*, Table).

In this report, we found several mismatches among viral sequences in primer/probe regions for molecular diagnosis. Such mismatches could lead to a lower sensitivity of the assay, further leading to false-negative diagnosis. The mismatched sequence data could have been generated by errors in PCR or sequencing during viral nucleotide sequence analysis because of incorporation of the wrong nucleotide [10]. However, it is more likely that the RNA virus has evolved and accidentally resulted in induction of mutation/s in the region targeted by the primer/probe for RT-PCR, only 2

87 years after establishment of the protocols. Fortunately, there are no or few mismatches for the most
88 screening assays for MERS-CoV. Still, protocols for molecular diagnosis of viral infections should be
89 reviewed regularly after establishment, particularly for viruses that pose a great threat to public
90 health, such as the MERS-CoV.
91
92 **Acknowledgement**
93 This research is supported by the Japan Initiative for Global Research Network on Infectious
94 Diseases (J-GRID) from Japan Agency for Medical Research and development, AMED. The funding
95 source had no involvement in the study design, in the collection, analysis and interpretation of data;
96 in the writing of the manuscript; and in the decision to submit the manuscript for publication.
97
98 **Conflict of interest statement**
99 All authors declare no conflict of interests.
100
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138 **Table**

139 Conservation of the primer and probe region sequences for the WHO-recommended assays for

140 molecular diagnosis of MERS-CoV.

141

Assay	Regions	Sequence (5′to 3′) *	Conservation [#]
UpE	forward primer	G 'C' AACGCGCGATTCAGTT	99% (115/116)
	reverse primer	GCCTCTACACGGGACCCATA	100% (115/115)
	probe	C 'T' CTTCACATAATCGCCCCGAGCTCG	99% (115/116)
ORF1a	forward primer	CCACTACTCCCATTTCGTCAG	100% (119/119)
	reverse primer	CAGTATGTGTAGTGCGCATATAAGCA	100% (119/119)
	probe	TTGCAAATTGGCTTGCCCCCACT	100% (119/119)
ORF1b	forward primer	TTCGATGTTGAGGGTGCTCAT	100% (116/116)
	reverse primer	TCACACCAGTTGAAAATCCTAATTG	100% (115/115)
	probe	CCCGTAATGCATGTGGCACCAATGT	100% (116/116)
N2	forward primer	GGCACTGAGGACCCACGTT	100% (127/127)
	reverse primer	TTG 'C' GACATACCCATAAAAGCA	99% (126/127)
	probe	CCCCAAATTGCTGAGCTTGCTCCTACA	100% (126/126)
N3	forward primer	GGGTGTACCTCTTAAT 'G' CCAATTC	95% (125/131)
	reverse primer	TCT 'G' TCCTGTCTCCGCCAAT	99% (130/131)
	probe	ACCCC 'T' GCGCAAAATGCTGGG	92% (120/131)
RdRpSeq	forward primer	TGCTATWAGTGCTAAGAATAGRGC	100% (119/119)
	reverse primer	GCATWGCNCW 'G' TCACACTTAGG	0% (0/110)
	corrected reverse primer	GCATWGCNCWATCACACTTAGG	100% (120/120)
	reverse-nested primer	CACTTAGGRTARTCCCAWCCCA	100% (120/120)
NSeq	forward primer	CCTTCGGTACAGTGGAGCCA	100% (127/127)
	reverse primer	GATGGG 'G' TT 'G' CCAAACACAAAC	49% (53/108)
	modified reverse primer	GATGGGRTTRCCAAACACAAAC	99% (107/108)
	forward-nested primer	TGACCCAAAGAATCCCAACTAC	100% (128/128)

142 * Position of mismatched nucleotide is indicated by quotation marks.

143 [#] (number of sequences perfectly matched/number of sequences available for the region)

144 WHO, World Health Organization; MERS–CoV, Middle East respiratory syndrome coronavirus

145 **Highlights**

146

147 • Protocols for laboratory diagnosis for MERS-CoV were developed in 2012.

148 • We analyzed nucleotide sequences of the viruses detected between 2012 and 2015.

149 • Mismatches between the sequences of primers/probes and viruses were found.

150 • Such mismatches could lead to a lower sensitivity of the assay.

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