

## Evidence of human coronavirus (229E), in patients with respiratory infection, Iran, 2015: the first report

Ali Madhi<sup>1</sup>, Arash Ghalyanchilangeroudi<sup>2\*</sup>, Mohammad Soleimani<sup>1</sup>

<sup>1</sup>Department of Microbiology, Faculty of Medicine, AJA University of Medical Sciences, Tehran, Iran

<sup>2</sup>Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

Received: May 2016, Accepted: July 2016

### ABSTRACT

**Background and Objectives:** Human coronaviruses (HCoVs) are one of the main causes of upper respiratory tract infections in humans. While more often responsible for mild illness, they have been associated with illnesses that require hospitalization.

**Materials and Methods:** 270 Samples from patients hospitalized with the respiratory infection during the autumn season of 2015 were evaluated for the presence of four HCoVs (OC43, 229E, HUK1, and NL63) using an optimized SYBR green RT-PCR assay.

**Results:** Fifteen HCoV-229E positive samples were identified (5.5 % positive). 85% of positive samples were male with the range of age between 12- 75 years old.

**Conclusion:** It is the first comprehensive study on determination of the role of human coronaviruses in respiratory infections in Iran. Our data provide a novel insight into the epidemiology of HCoVs in Iran. Further studies are needed and should include the isolation and molecular characterization of HCoVs in Iran.

**Keywords:** Human coronavirus, 229E, Iran, OC43, NL63 , HUK-1

### INTRODUCTION

Coronaviruses are globally distributed , although there are differences in the frequency of detection of the four viruses in different parts of the world at different times (1). Coronaviruses, a genus of the Coro-

naviridae family, are enveloped viruses with a large plus-strand RNA genome. The genomic RNA is 27–32 kb in size, capped and polyadenylated. Three serologically distinct groups of coronaviruses have been described. Within each group, viruses are characterized by their host range and genome sequence. Coronaviruses have been identified in mice, rats, chickens, turkeys, swine, dogs, cats, rabbits, horses, cattle and humans, and can cause a variety of severe diseases including gastroenteritis and respiratory tract diseases (2). In humans, coronaviruses are main causes of respiratory tract infections, whereas, in animals, they can cause respiratory, enteric, hepatic and neurological diseases of varying severity (3). Hu-

\*Corresponding author: Arash Ghalyanchi Langeroudi, Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Azadi Ave , Tehran, Iran. PO.Cod:1419963111.  
Tel: 021-61117154  
Fax: 021-66933222  
Email: arashghalyanchi@gmail.com

man coronaviruses (HCoVs) have attracted renewed interest recently, in part because of the emergence of a novel HCoV associated with the severe acute respiratory syndrome (SARS). Before the emergence of CoV-SARS, HCoVs were generally thought to cause mild, self-limited infections of the upper respiratory tract. The study of HCoVs has been hampered by the difficulty in propagating these viruses *in vitro* (4). First identified in the mid-1960s, HCoV-229E and HCoV-OC43 are recognized to be an important cause of the common cold. Epidemiologic studies have since shown that these viruses are responsible for up to 30% of mild upper respiratory tract infections. More recently, 2 new HCoVs, NL63 and HKU1, have been reported, both detected in respiratory tract specimens from patients with lower respiratory tract illness (5). The complete viral genome sequence was determined. The characteristic genome organization of coronaviruses can be observed: the 5' two-third of the genome contains two large open reading frames (ORF), ORF1a and ORF1b. In the 3' part of the genome, genes encoding four structural proteins are found: spike (S), envelope (E), membrane (M), and nucleocapsid (N). The hemagglutinin-esterase (HE) gene, characteristic of group 2 coronaviruses, is not present in HCoV-NL63 (6). HCoVs are recognized as one of the most frequent causes of URTI or common colds in adults, epidemiological data, and clinical profiles are limited to HCoVs infection in adults with URTI continuously for several years with sensitive molecular methods. There is a need for rapid, sensitive, and accurate diagnosis of lower respiratory tract infections in children, elderly, and immunocompromised patients, who are susceptible to serious complications (7). Few detection methods of coronavirus are available at present. Consequently, these viruses are sought rarely in diagnostic laboratories, and the associated clinical symptoms associated are not defined. Some molecular detection methods were described recently for screening for HCoVs: PCR amplification, simple or nested, with primers defined in the N protein gene (8). The aim of this study was determinations of role of HCoVs in some respiratory infections in Iranian patients during autumn, 2015.

## MATERIALS AND METHODS

**Sampling.** 270 nasopharyngeal swabs from suspected patients that show respiratory clinical signs

were collected during October to December 2015 (9). Samples were collected from three hospitals in Tehran city (Besat, Golestan, and Imam REZA). The patients suffered from respiratory syndrome such as coughing, gasping, and excessive lacrimation. The range of age was between 12-75 years old. In addition, the males and females were 65% & 35% of the cases respectively.

**RNA Extraction.** RNA was extracted from swabbed samples using high pure viral RNA kit (Roche Co., Germany) according to manufacturer's instructions. The purity of the extracted RNA was determined by taking the ratio of the readings at 260 and 280 nm. We used negative control during the extraction procedure.

**cDNA synthesis.** For cDNA synthesis, 1  $\mu$ L (0.2  $\mu$ g) of random hexamer primer (SinaClon, Iran) was added to 5  $\mu$ L of extracted RNA and the mixture was heated at 65°C for 5 minutes. Fourteen  $\mu$ L of cDNA master mix containing 7.25  $\mu$ L DEPC-treated water (SinaClon, Iran), 2  $\mu$ L dNTP mix (SinaClon, Iran), 0.25  $\mu$ L RiboLock RNase Inhibitor (Thermo Fisher Scientific, USA), 0.5  $\mu$ L Revert Aid Reverse Transcriptase (Thermo Fisher Scientific, USA), and 4  $\mu$ L 5X RT Reaction Buffer was added to each tube, resulting in a final volume of 20  $\mu$ L. Then, the mixture was incubated at 25°C for 5 min, 42°C for 60 min, 95°C for 5 min, and 4°C for 1 min, respectively. The cDNA was stored at -20°C until use (10).

**SYBR green Real-Time PCR.** cDNA was amplified by a real-time PCR using Real Q PCR 2 x master mix (Ampliqon), in the Rotor-Gene Q (Qiagene, USA). The primers sequences are available in Table 1. Each reaction had a total volume of 25  $\mu$ L, including 12.5  $\mu$ L SYBR Green master mix, 200 nmol of each forward and reverse primers, 5  $\mu$ L cDNA plus 7.1  $\mu$ L ddH<sub>2</sub>O. The cycling conditions included an initial denaturation step of 10 minutes at 94°C, followed by 40 cycles of 15 seconds at 95°C, one minute at 55°C and one minute at 60°C. Fluorescent detection was at the end of each cycle. Melting curve analysis program was used for identification of specific PCR products. After the last cycle, the temperature was increased to 94°C, then decreased to 75°C and slowly increased to 94°C at a rate of 0.1°C per second, with continuous fluorescence monitoring. Positive and negative controls were added in each set

**Table 1.** Primers used in detection of human coronaviruses

Human Coronavirus	Primer Name	Sequence (5'-----> 3')	Gene target	References
		CGATGAGGCTATTCCGACTAGGT		
OC43	OC43F OC43R	CCTTCCTGAGCCTTCAATATAGTAACC ACGTACTTCTATTATGAAGCATGATATTAA	Nucleocapsid	76 (25)
NL63	NL63F NL63R	AGCAGATCTAATGTTATACTTAAAACACTACG CAGTCAAATGGGCTGATGCA	Nucleoprotein	103 (25)
229E	229E-F 229E-R	AAAGGGCTATAAAGAGAATAAGGTATTCT CCTTGCGAATGAATGTGCT	Nucleoprotein	103 (25)
HUK1	HUK1-F HUK1-R	TTGCATCACCCTGCTAGTACCAC	Replicase 1b	95 (5)

(11). For final confirmation, the PCR product was analyzed by electrophoresis on 2.5% agarose gel and visualized under UV light.

## RESULTS

In the set of coronaviruses NL63, 229E, OC43, HUK-1 the only detected virus was 229E (5.5%, is positive samples). Of positive samples for all three months, 85% were male with the range of age between 12- 75 years old.

## DISCUSSION

This study presents the first evidence that HCoV 229E circulates in patients with respiratory infections in Iran. Based on the serologic and phylogenetic relationship, coronaviruses are classified into four genera. Alphacoronavirus and betacoronavirus consist of various mammalian coronaviruses, whereas gammacoronavirus and delta coronaviruses include avian viruses. The genus of Alphacoronavirus includes transmissible gastroenteritis virus (also referred to as alphacoronavirus I; ICTV 2009), porcine epidemic diarrhea virus, some bat coronaviruses, and the HCoV-OC43, NL63 and 229E. In January 2005, a new group betacoronavirus, HCoV-HKU1, was found in 2 patients with pneumonia in Hong Kong. In general, HCoV-229E virus causes common cold but occasionally it can be associated with more severe respiratory infections in children, elderly and persons with underlying illness (12-14). HCoVs are increasingly recognized as respiratory pathogens associated with a broadening range of clinical outcomes, whereas they were once recog-

nized as “common cold” viruses. Although, Gaunt et al (2010) demonstrate that HCoV-OC43, HCoVNL63, and HCoV-HKU1 were specifically associated with lower respiratory tract disease in the study population (1). Nasopharyngeal sampling was shown to have advantages over oropharyngeal sampling for the identification of coronaviruses in previous studies (9). Several reports on Human coronaviruses and other respiratory viruses have been done in Iran. Sultani et al (2015) designed multiplex SYBR Green Real-Time PCR assay for detection of respiratory viruses including HCoVs. Application of the multiplex SYBR Green real-time PCR in clinical samples from 172 patients in a one-year study resulted in detection of 19 (11.04%) PIV3, 9 (5.23%) PIV4, and 1 (0.58%) coronavirus NL63. All the positive samples were detected during December to March (2011-2012) (11). They detected coronavirus NL63 in a newborn with the acute respiratory infection which shows that HCoVs can lead to the severe respiratory infections and hospitalization as the first time (15). During January 2013–August 2014, a total of 1,800 patients in Iran who had respiratory illness were tested for MERS-CoV. Patients tested during 2013 had been pilgrims to Mecca, Saudi Arabia, during the Hajj; patients tested during 2014 were pilgrims or had been hospitalized for respiratory infections with unknown causes. Yavarian et al (2015) report a cluster of 5 cases that occurred in the same hospital in Kerman Province, Iran, during May–July 2014 (16). In other study by Alborzi et al (2009) on Iranian Hajj pilgrims with symptoms of acute respiratory tract infections, they show eighty-three patients (32.5%) had viral pathogens: influenza in 25 (9.8%), parainfluenza in 19 (7.4%), rhinovirus in 15 (5.9%), adenovirus in 14 (5.4%), enterovirus in 5 (2%), and RSV in 4 (1.6%) and coinfection with two viruses

in 1 patient (0.4%) (17). Recently, some Iranian researchers reported Metahumanpenumovirus (18, 19) and Human Bocavirus (20) in patients with acute respiratory infections. In addition, the role of Respiratory syncytialviral infection were confirmed in Iran (21-23). In study of Lu et al (2012), they detected 4 non-SARS related HCoV species by real-time RT-PCR in 981 nasopharyngeal swabs collected from March 2009 to February 2011. The species detected were 229E (96 cases, 9.8%), OC43 (42 cases, 4.3%), HKU1 (16 cases, 1.6%) and NL63 (11 cases, 1.1%). HCoV-229E was circulated in 21 of the 24 months of surveillance (24).

Gaunt et al (2010) conducted a large-scale comprehensive screening for all four coronaviruses by analysis of 11,661 diagnostic respiratory samples collected in Edinburgh, United Kingdom, over 3 years between July 2006 and June 2009 using a novel four-way multiplex real-time reverse transcription-PCR (RT-PCR) assay. Coronaviruses were detected in 0.3 to 0.85% of samples in all age groups. Generally, coronaviruses displayed marked winter seasonality between the months of December and April and were not detected in summer months, which is comparable to the pattern seen with influenza viruses. HCoV-229E was the exception; detection was confined to the winter of 2008 and was sporadic in the following year (1). Dare et al (2007) developed a real-time reverse-transcription polymerase chain reaction (RT-PCR) assay panel for the recognized HCoV types and compared HCoV infections in patients hospitalized with pneumonia, outpatients with influenza-like illness, and asymptomatic control patients between September 2003 and August 2005. During the study year 1, 43 (5.9%) of 734 patients with pneumonia had HCoV infections; 72.1% of the infections were with OC43. During the second year of study, when control patients were available, 21 (1.8%) of 1156 patients with pneumonia, 12 (2.3%) of 513 outpatients, and 6 (2.1%) of 281 control patients had HCoV infections (5). The results, however, indicate that HCoV is detected and that the novel real-time RT-PCR assay provides a tool for large-scale epidemiological studies to further clarify the role that coronavirus infection plays in respiratory infections in humans. Our data provide a novel insight into the epidemiology and clinical knowledge of HCoVs in Iran. Further studies are needed and should include the isolation and molecular characterization of HCoVs in Iran. However, because year-to-year variation in overall prevalence and type of circulating HCoV can

occur, a multiyear study with appropriate control subjects will be necessary to adequately assess whether there is an association between HCoV infection and respiratory disease.

#### ACKNOWLEDGEMENT

This study was conducted under grant of research council, AJA University of medical sciences and IVO grant.

#### REFERENCES

1. Gaunt E, Hardie A, Claas E, Simmonds P, Templeton K. Epidemiology and clinical presentations of the four human coronaviruses 229E, HKU1, NL63, and OC43 detected over 3 years using a novel multiplex real-time PCR method. *J Clin Microbiol* 2010;48(8):2940-7.
2. van der Hoek L, Pyrc K, Jebbink MF, Vermeulen-Oost W, Berkhout RJ, Wolthers KC, et al. Identification of a new human coronavirus. *Nat Med* 2004;10(4):368-73.
3. Woo PC, Lau SK, Yip CC, Huang Y, Yuen K-Y. More and more coronaviruses: human coronavirus HKU1. *Viruses* 2009;1(1):57-71.
4. Esper F, Shapiro ED, Weibel C, Ferguson D, Landry ML, Kahn JS. Association between a novel human coronavirus and Kawasaki disease. *J Infect Dis* 2005;191(4):499-502.
5. Dare RK, Fry AM, Chittaganpitch M, Sawanpanyalert P, Olsen SJ, Erdman DD. Human coronavirus infections in rural Thailand: a comprehensive study using real-time reverse-transcription polymerase chain reaction assays. *J Infect Dis* 2007;196(9):1321-8.
6. Moës E, Vijgen L, Keyaerts E, Zlateva K, Li S, Maes P, et al. A novel pancoronavirus RT-PCR assay: frequent detection of human coronavirus NL63 in children hospitalized with respiratory tract infections in Belgium. *BMC Infect Dis* 2005 Feb;1;5:6.
7. Coiras M, Aguilar J, Garcia M, Casas I, Pérez-Breña P. Simultaneous detection of fourteen respiratory viruses in clinical specimens by two multiplex reverse transcription nested-PCR assays. *J Med Virol* 2004;72(3):484-95.
8. Vabret A, Mouthon F, Mourez T, Gouarin S, Petitjean J, Freymuth F. Direct diagnosis of human respiratory coronaviruses 229E and OC43 by the polymerase chain reaction. *J Virol Methods* 2001;97(1):59-66.
9. Lieberman D, Lieberman D, Shimoni A, Keren-Naus A, Steinberg R, Shemer-Avni Y. Identification of respiratory viruses in adults: nasopharyngeal versus oropharyngeal.

- ryngeal sampling. *J Clin Microbiol* 2009;47(11):3439-43.
10. Najafi H, Langeroudi A, Hashemzadeh M, Karimi V, Madadgar O, Ghafouri S, et al. Molecular characterization of infectious bronchitis viruses isolated from broiler chicken farms in Iran, 2014-2015. *Arch Virol* 2016 Jan;161(1):53-62.
  11. Sultani M, Azad TM, Eshragian M, Shadab A, Naseri M, Eilami O, et al. Multiplex SYBR green real-Time PCR assay for detection of respiratory viruses. *Jundishapur J Microbiol* 2015 Aug 1;8(8):e19041.
  12. Farsani SMJ, Dijkman R, Jebbink MF, Goossens H, Ieven M, Deijns M, et al. The first complete genome sequences of clinical isolates of human coronavirus 229E. *Virus genes* 2012;45(3):433-9.
  13. van Elden LJ, van Alphen F, Hendriksen KA, Hoepelman AI, van Kraaij MG, Oosterheert J-J, et al. Frequent detection of human coronaviruses in clinical specimens from patients with respiratory tract infection by use of a novel real-time reverse-transcriptase polymerase chain reaction. *J Infect Dis* 2004;189(4):652-7.
  14. Vabret A, Dina J, Gouarin S, Petitjean J, Corbet S, Freymuth F. Detection of the new human coronavirus HKU1: a report of 6 cases. *Clin Infect Dis* 2006;42(5):634-9.
  15. Sultani M, Mokhtari-Azad T, Yavarian J. Detection of human coronavirus NL63 in a 28 Days old newborn in Iran. 2. [Original article]. 2013;7(3):27-8.
  16. Yavarian J, Rezaei F, Shadab A, Soroush M, Gooya MM, Azad TM. Cluster of Middle East respiratory syndrome coronavirus infections in Iran, 2014. *Emerg Infect Dis* 2015;21:362-364.
  17. Alborzi A, Aelami MH, Ziyaeyan M, Jamalidoust M, Moeini M, Pourabbas B, et al. Viral etiology of acute respiratory infections among Iranian Hajj pilgrims, 2006. *J Travel Med* 2009;16:239-242.
  18. Moattari A, Aleyasin S, Arabpour M, Sadeghi S. Prevalence of human Metapneumovirus (hMPV) in children with wheezing in Shiraz-Iran. *Iran J Allergy Asthma Immunol* 2010 Dec;9(4):250-254.
  19. Arabpour M, Samarbafzadeh A, Makvandi M, Shamsizadeh A, Percivalle E, Englund J, et al. The highest prevalence of human metapneumovirus in Ahwaz children accompanied by acute respiratory infections. *Indian J Med Microbiol* 2008;26:123.
  20. Naghipour M, Cuevas LE, Bakhshinejad T, Dove W, Hart CA. Human bocavirus in Iranian children with acute respiratory infections. *J Med Virol* 2007;79:539-543.
  21. Milani M. Respiratory syncytial virus infection among young children with acute respiratory infection. *Acta Med Iran* 2003;41(4):269-72.
  22. Farshad N, Saffar M, Khalilian A, Saffar H. Respiratory viruses in hospitalized children with acute lower respiratory tract infections, Mazandaran Province, Iran. *Indian Pediatr* 2008;45(7):590.
  23. Faghihloo E, Rezaie F, Salimi V, Naseri M, Mamishi S, Mahmoodi M, et al. Molecular epidemiology of human respiratory syncytial virus in Iran. *Acta Virol* 2011;55(1):81-83.
  24. Lu R, Yu X, Wang W, Duan X, Zhang L, Zhou W, et al. Characterization of human coronavirus etiology in Chinese adults with acute upper respiratory tract infection by real-time RT-PCR assays. *PloS one* 2012;7(6):e38638.
  25. Gunson R, Collins T, Carman W. Real-time RT-PCR detection of 12 respiratory viral infections in four triplex reactions. *J Clin Virol* 2005;33(4):341-4.