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Detection and genotyping of rhinovirus from exacerbated asthmatic patients in Baghdad, Iraq

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Asthma is a chronic disease with multiple environmental and genetic causes. Determining the role of rhinovirus in asthma incidence and exacerbation could improve the controlling measures for this disease. This study aims to detect and genotype human rhinovirus (HRV) in asthmatic patients in Iraqi population. A total of 45 patients with asthma participated in this study. Viral RNA was extracted from nasopharyngeal swabs (NPS) and cDNA was created using reverse transcriptase-polymerase chain reaction (PCR). Specific primers for HRV were used with two rounds nested-PCR to amplify the 5'-noncoding region of the viral genome. PCR products of the second round nested-PCR underwent direct sequencing and the resultant sequences were aligned with reference sequences in GenBank. MEGA 5 software was used to construct phylogenetic tree between eight successfully sequenced isolates and eight reference isolates. Alignment of viral sequence revealed highly genetic diversity between these sequences and the reference isolates. Phylogenetic tree showed that five isolates belong to Human Rhinovirus- A (HRV-A), while three isolate belong to Human Rhinovirus-C (HRV-C). The HRV-C was detected and genotyped for the first time in Iraq. The results of the current study suggest the significant role of HRV infection among patients with exacerbated asthma in Iraq.

Key words: Human rhinovirus, genotyping, asthma exacerbation.

INTRODUCTION

Asthma is a very common chronic condition affecting all age classes especially children. Approximately, 235 million people are affected with asthma worldwide, with an unusual death figure of 250000 (WHO, 2011). Not

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only is the disease considered a big public health problem, but also the burden economic cost is very high. World Health Organization (WHO) estimated that the economic cost of asthma exceeds the cumulative cost of human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) and tuberculosis (WHO, 2004, 2011).

Asthma exacerbation may be caused by both genetic and environmental factors. Infectious agents are among the most important environmental factors that influence asthma exacerbation (Guilbert and Deulnger, 2010). Despite the fact that the respiratory system is a target for large numbers of bacteria, fungi, and even parasites, viruses seem to have more important role in asthma than other microorganisms. HRV has been noted as pathogens of the common cold for over 50 years; however, recent advances in viral molecular diagnostics have brought the attention for more significant role of these viruses in respiratory diseases (Arakawa et al., 2012; Moore et al., 2013).

HRVs are a group of single strands RNA positive sense, non-enveloped viruses, that are protected by an icosahedral proteineous capsid. They belong to the genus *Enterovirus* and Picornaviridae family (Pallansch and Roos, 2007). The viral RNA composes of about 7.2 kb and consists of a single gene that produces 2 proteins (Simmonds et al., 2010). The single open reading frame (ORF) has three regions: the first region (P1) encodes for the structural proteins, while the other two regions (P2 and P3) encode for the non-structural proteins (Jacobs et al., 2013). In the 5'-non-coding region (5'-NCR), there is located an internal ribosomal entry site (IREs) which is necessary for translation (Langereis et al., 2014).

HRVs were originally classified into two species (A and B). Due to the highly sensitive molecule methods, a novel species (HRV-C) was identified and designed (Lamson et al., 2006). The new species has genomic organization similar to the other two species; however, several distinct differences gave it a new classification. More than 50 different types of HRV-C have been identified on the basis of 13% nucleotide differences in VP1 encoding gene (Bochkov and Gern, 2012). To the best of the authors' knowledge, there is no previous study that investigated the most prevalent species of these viruses among Iraqi population. Thus, the current study aims to determine HRVs species among asthmatic patients.

METHODOLOGY

Study population

A total of 45 asthmatic adult patients with exacerbation were included in the study. Age range was from 16 to 48 years, 18 (40%) males and 27 (60%) females. These patients were attending Al Zahra'a Consultative Center for Allergy and Asthma, and Consultative Clinic for Chest and Respiratory Diseases/Baghdad, from January to April 2015. The diagnosis of asthma was performed by a specialist physician. The exclusion criteria were any history of respiratory illness like chronic obstructive pulmonary disease (COPD), tuberculosis, pneumonia, or bronchitis, and any other comorbid illness such as diabetes mellitus, hypertension, or pregnant females. Informed consent was obtained from all participants and ethical approval was obtained from the ethical committee of Colleges of Medicine, Al-Nahrain University.

Nasopharyngeal swabs

Nasopharyngeal swabs (NPS) were collected using the flexible flocked swab with personal protective equipment. The swab was gently entered along the base of one nostril (straight back, not upwards) and continued along the floor of the nasal passage until it reached the nasopharynx. After 2 to 3 times rotation, the swab was held in place for 5 s. A suitable tube with cover was used to keep the swab. The excess length of swab was broken off at the score mark to permit capping of the tube and transported with ice packs to the laboratory for processing. Time duration from swab collection to laboratory processing did not exceed an hour in any case.

Viral RNA extraction and cDNA synthesis

A ready kit (QIAampMinElute Virus Spin Kit, Germany) was used for RNA extraction from NPSs. The kit combines the selective binding properties of a silica-based membrane with flexible elution volumes of between 20 and 150 µl. The instruction manual of the company was followed precisely, with an eventual elution volume of 50 µl. Another ready kit (iNtRON Power cDNA Synthesis Kit/Korea) was used for reverse transcriptase-polymerase chain reaction (RT-PCR) according to the manufacturer's instructions.

HRV genotyping

Resultant cDNA samples were prepared to perform the genotyping study. Semi-nested PCR was used for the amplification of 5'-noncoding region of HRV genome. The primer pair for the first round was forward: P1-1 5'-CAAGCACTTCTGTYWCCCC and reverse: P3-1 5'-ACGGACACCCAAAGTAG. The PCR conditions were as follows: after an initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 45 s and elongation at 72°C for 1 min. These cycles were followed by final elongation at 72°C for 7 min. This round gives an amplicon with 390 bp which belongs to the 5'-NCR of all species of HRV.

In the second round, the same forward primer was used while three types of primers were simultaneously used as reverse primers. These were P2-1: TTAGCCACATTCAGGGGC, P2-2: TTAGCCACATTCAGGAGCC and P2-3: TTAGCCGCATTCAGGGG. This variation in primers was to ensure the amplification of all three species of HRV. The same above cycling conditions were applied except for annealing temperature which was 61°C instead of 58°C.

Out of 45 NPAs, only nine gave PCR product in the first and second round semi-nested PCR. These products were directly sequenced by Sandor lifescience Pvt. Ltd/India, using the ABI Big Dye Terminator v.3.0. Products are resolved by electrophoresis on an ABI 3730xl capillary sequencer.

The resultant sequences were undergone BLAST in National Center for Biotechnology Information (NCBI). Accordingly, eight close sequences were chosen to represent the three species of HRV. Furthermore, the sequences and chosen reference sequences were aligned together using Bioedit software. To identify the species of each isolate, a phylogenetic tree with 1000 bootstrap replicates was constructed using MEGA5 software.

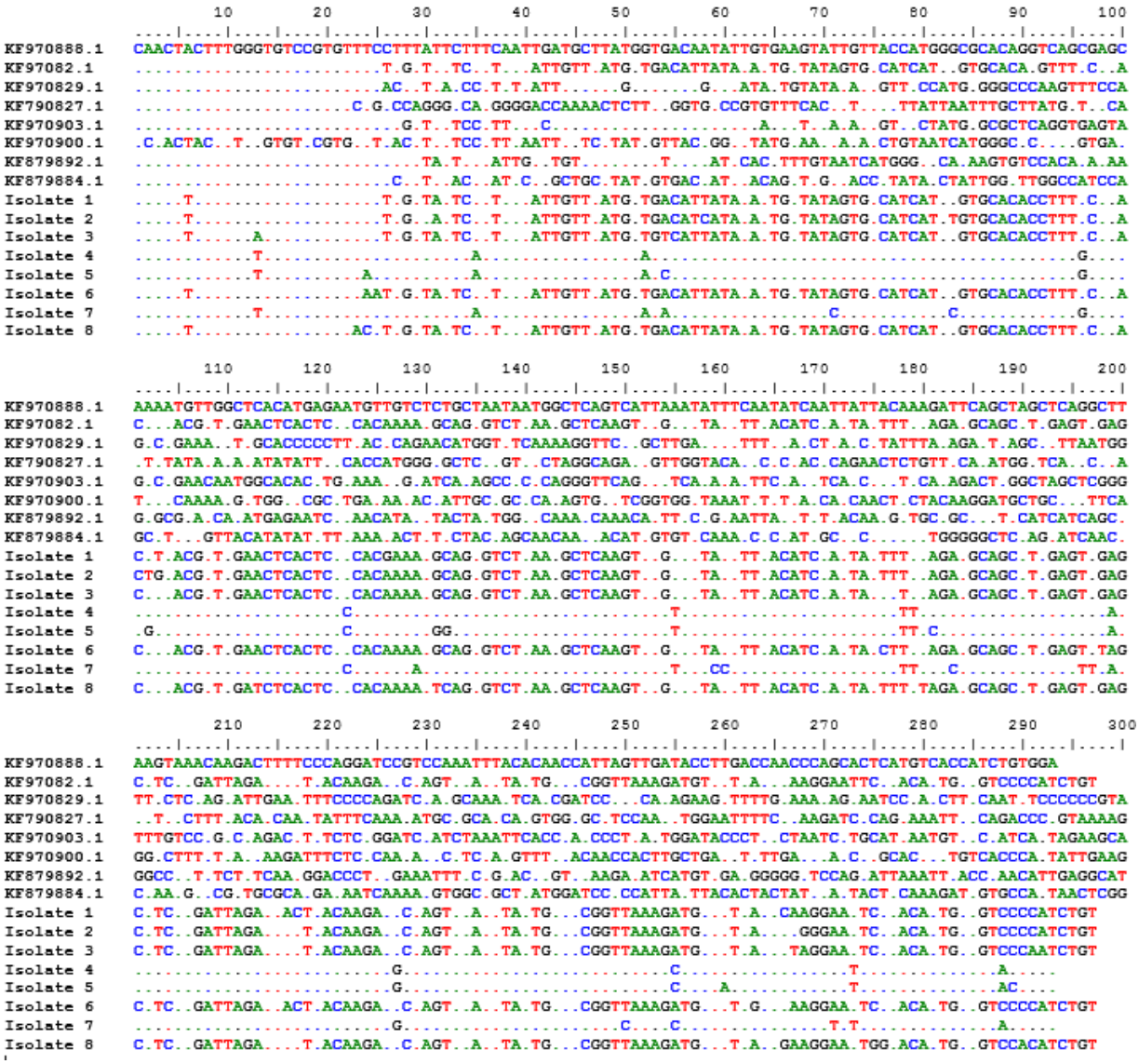

Figure 1. Alignment of the eight isolates with reference sequences from National Center for Biotechnology Information using Bioedit software.

RESULTS

Human Rhinovirus detection and genotyping

Viral RNA was successfully extracted from 9 out of 45 NPSs (20%). cDNA was created from extracted RNA. The resultant cDNA was amplified by semi-nested PCR using specific primers for the first round and second round PCR. Both rounds ended with successful amplification of the target gene.

Sequence alignment

From the nine positive samples for HRV, only eight were successfully sequenced. These sequences underwent BLAST in NCBI web page from which eight similar sequences were chosen for the alignment and for construction of phylogenetic tree with the isolates of the current study. The accession numbers of reference sequences were KF97082.1, KF970829.1 and KF970827.1 representing HRV-A, KF879892.1 and KF879884.1 representing HRV-B and KF970888.1; KF970903.1 and KF970900.1 representing HRV-C. Figure 1 shows the alignment results using BioEdit soft. It is obvious from the figure the high diversity among the isolates. A preliminary reading for this alignment reveals that the isolates number 4, 5 and 7 were very close to the reference sequence KF970888.1 (HRV-C), while the isolates numbers 1, 2, 3, 6 and 8 were very close to the

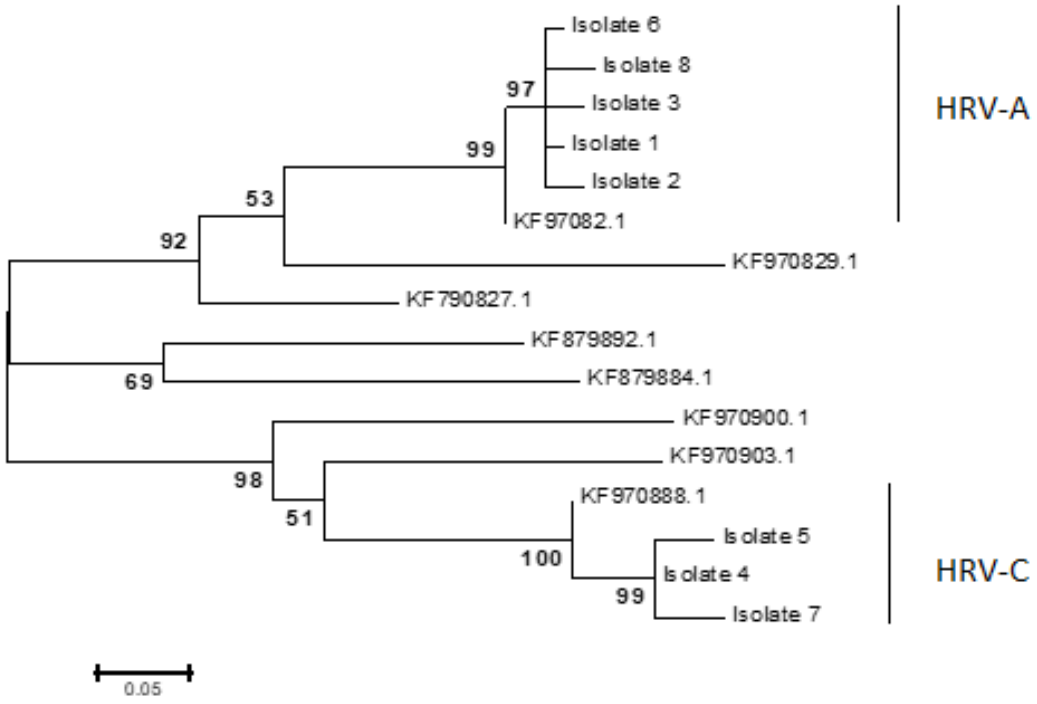

Figure 2. Phylogenetic tree based on 5'-non coding region constructed using maximum likelihood method with MEGA 5 software.

reference sequence KF97082.1 (HRV-A), although there are some substitutions in several nucleotides.

Figure 2 shows the phylogenetic tree for the 8 isolates and the reference sequences. The results of this phylogenetic analysis absolutely show that the isolates 1, 2, 3, 6 and 8 belong to HRV-A, while the isolates 4, 5 and 7 belong to HRV-C.

DISCUSSION

The current study revealed that 9 (20%) of the exacerbated asthmatic patients out of 45 were infected with HRV. Approximately close results were recorded in different parts of the world. In Hong Kong, Susanna et al. (2009) used RT-PCR for detection of HRV in asthmatic patients. They found that 220 NPS out of 1200 (18.3%) were positive for HRV. In China, Sun et al. (2016) reported that 14.7% of 709 hospitalized asthmatic patients have HRV. Very large percentage, however, was reported by some studies even with 60% in case of exacerbated asthma (Johnston et al., 2007). The difference in the percentage of HRV between the current study and the aforementioned studies may be partly explained by sample size, which becomes more representative when a high number of patients are involved, and based on the seasons at which the studies are performed. In this regard, studies conducted in cold and humid season have greater opportunity to find the virus as compared to that conducted in hot and dry seasons (Johnston et al., 2005; Leotte et al., 2017).

To the best of the authors' knowledge, this is the first study in Iraq regarding detection of HRV-C by molecular method. In fact, two species of HRV were detected among asthmatic patients. Among the 9 positive asthmatic patients, HRV-A was detected in 5 samples (55.5%) and HRV-C in 3 samples (33.3%), while the sequence in one sample could not be detected.

Most previous studies in this regard reported high prevalence of HRV-A and HRV-C with low prevalence of HRV-B in asthmatic patients. Lau et al. (2009), in Hong Kong, found that 21 out of 26 (81%) of HRV- positive nasopharyngeal aspirates (NPAs) belong to HRV-C and only 5 (19%) belong to HRV-A; while HRV-B was not detected. The first report from the Middle East (Jordan) pointed out that 26% of HRV- positive NPAs from asthmatic patients were related to HRV-C (Miller et al., 2009a). In Hong Kong, HRV-A, HRV-C and HRV-B represented 50, 41 and 8% respectively (Lau et al., 2009). However, this percentage outbalanced towards HRV-C which accounts for 50% of HRV- samples in the United State (Miller et al., 2009b). Equal or more percentages for HRV-C were reported in many other countries (Lamson et al., 2006; Renwick et al., 2007; Briese et al., 2008).

In fact, such disparities are expected depending on many factors, the most important of which is age of the patients. HRV-C is usually more associated with children

with asthma. Lau et al. (2009) demonstrated that wheezing episodes were more frequent in children infected with HRV-C than either HRV-A or HRV-B. Most studies recorded higher prevalence of HRV-C than HRV-A which were conducted on hospitalized patients suffering from asthma episode; while in the current study, samples were taken from out-patients. On the other hand, most adults with HRV-C had underlying diseases. Majority of the patients in the current study are adults, thus relatively low prevalence of HRV-C occurred. This tendency of HRV-C is not fully understood. It may be related to the immune status of individual. The second factor is the severity of asthma episode. HRV-C is more common in asthma exacerbation (Liao et al., 2016).

5'-Noncoding region and VP4/VP2 region are the most commonly used target for genotyping of HRV-C with 5'-NCR is more sensitive (Kiang et al., 2008; Han et al., 2009). Sequencing of short amplicon of this region (270 to 290 bp) is usually enough for strain typing (Lee et al., 2007). The analysis of alignment of these sequences revealed that the highly conserved HRV sequence is present only in 5'-NCR. In the current study, universal primers were used to anneal this highly conserved motif and PCR products were directly sequenced and aligned with the closest serotypes according to the GenBank database. The results of this alignment showed the high diversity among the different strains. In fact, such diversities were frequently reported. Daleno et al. (2013) found that nucleotide variability between HRV-A and HRV-C may reach 37%. The variability is not only found between different species, but also within strains that belong to the same species. Arakawa et al. (2012) showed that HRV-C genomes have more than 30% divergence. Furthermore, McIntyre et al. (2010) suggested that there are approximately 30 genotypes belonging to HRV-C based on diversity in nuclear acid sequence. However, Lee et al. (2007), in a previous study, reported 94 to 100% identity among HRV-C strains.

In phylogenetic analysis, the 8 isolates of HRVs were clustered into 2 different clusters; one near the strains which belong to HRV-A and the other cluster near the strains which belong to HRV-C. The relatively high numbers of bootstrap in the nodes indicate that these strains belong to the corresponding neighboring reference sequences.

HRV does not cause cytopathic effect, and the limited epithelial distribution is not correlated with the severity of exacerbated asthma. These facts suggest alternative mechanism(s) by which the virus induces asthma exacerbation. The current hypothesis assumes that HRV causes the release of epithelial cells of broad spectrum of chemokine's such as CXCL, CCL5 and CXCL10, which attract the inflammatory cells to the airways (Johnston et al., 2005). Edwards et al. (2012) suggested the pre-request of previous sensitization in the respiratory epithelial cells in order for HRV to cause exacerbation. Sensitization promotes Th2 cells and HRV stimulate these cells to release different cytokines and chemokines. These in turn attract airways more to Th2 cells which secrete three important cytokines: IL-5, IL-4 and IL-13. IL-5 promotes eosinophilia which results in increase in eosinophil cationic protein (ECP) and transforming growth factor β (TGF-β) and eventual inflammation of airway smooth muscle. IL-4 and IL-13 cause antibody class switching to IgE which binds mast cells. The cross linking of allergen on mast cell-bound IgE causes mast cell degranulation and release of profound mediators such as histamine, prostaglandin and leukotrienes. These mediators cause bronchoconstriction and further airway inflammation (Reuter et al., 2010). These data indicate the significant role of rhinovirus in asthma, and it seems that the vast majority of HRV species in Iraqi patients with asthma are HRV-A and HRV-C.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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