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First identification of Human Bocavirus (HBoV) in Iraqi children with respiratory complications

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Abstract : *Due to lacking of previous studies in Iraq, this study was conducted and aimed to detect the HBoV DNA in children infected with flu-like illness by real-time PCR technique. A total of 195 nasopharyngeal swabs samples were collected from children suffering from influenza like illnesses (ILI), aged 15 years and less (one month – 15 years), samples were collected from public hospitals at different Iraqi provinces in the period from September 2015 to May 2016. All clinical samples tested with real-time PCR technique for detection. Results showed 48/195 (24.62%) positive case for HBoV with threshold (Ct) values ranged from 15.25 to 38.7 (32.29 ± 6.5). Moreover,* the *high virus load was noted mainly in the absence of other testing respiratory viruses and suggesting a causative role for HBoV. However, low virus load were more detected in virus co-infection. Finding found that age < 2 years and high virus load variable were associated with severe LRTI, while virus co-infection would not increase disease severity. Therefore the more severe lower respiratory tract symptom presented in high HBoV virus load patients may solely depend on HBoV virus load. In conclusion, Human bocavirus have been recognized and reported as the etiologic agents of respiratory tract infections predominantly in children, the distinguishing proof of new viruses will keep on occurring as molecular methodology tests turn out to be more contemporary. No further study was ever done to detect HBoV in Iraq, by far, this is the first record, and the key reference for further studies over it.*

Keywords: *Human Bocavirus, hMPV, RSV, Real-Time PCR, Respiratory viral infections, Iraqi children.*

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I. Introduction

Acute respiratory tract infections are responsible for considerable morbidity and mortality in humans, and the costs attributable to acute respiratory tract illnesses (RTIs) are an important burden on national health care budgets. Most of the viruses belonging to the families of Paramyxoviridae (human Metapneumovirus, Human Parainfluenza Viruses, Respiratory Syncytial Virus), Orthomyxoviridae (Influenza viruses, Avian Influenza Virus), Picornaviridae (Encephalomyocarditis Virus, Human Parechoviruses, Human Rhinoviruses A, B, and C), Adenoviridae (Adenoviruses), Parvoviridae (Human Bocavirus) and Coronaviridae (Human Coronaviruses) are contributed in respiratory complications [1]. When the first human bocavirus, (HBoV1) was discovered, it was found to be related to bovine parvovirus (BPV) and minute virus of canines (MVC), the original members of the genus *Bocavirus*, hence the name human bocavirus; and it shows that HBoV-1 has an ancient zoonotic origin [2]. Soon after, three other genotypes (HBoV 2-4) were identified in this genus. While HBoV1 was commonly associated with RTIs, HBoV2-4 was isolated from fecal samples [3-6].

Epidemiological studies conducted in different countries and various age groups have demonstrated that the detection rate of HBoV in patients with respiratory tract infections varies between 1.5%-18.3%, and it is detected more commonly in children less than 5 years of age. It causes respiratory tract infections leading to hospitalization in this group [7, 8] Other respiratory tract viruses, including RSV and adenovirus, are frequently involved in the infections caused by HBoV and may cause co-infections [9].

Due to the potential role of Respiratory viruses in the URTIs and LRTIs cases of young children and because of the lacking of previous studies in Iraq, this study was conducted and aimed to detect the HBoV DNA in children infected with flu-like illness by real-time PCR technique.

This technique has the ability to detect 1-1000 ng viral RNA and/or DNA, it does not depend on intact viral particles [10, 11] and thus this assay is performed to identify the hMPVs, RSVs in previous study [12] and HBoVs infections in children under the age of 15 years in this study.

II. Materials and Methods

2.1 Patients and Samples

A total of 195 nasopharyngeal swabs samples were collected from children suffering from influenza like illnesses (ILI), aged 15 years and less (one month – 15 years), samples were collected from public hospitals at different Iraqi provinces in the period from September 2015 to May 2016. All samples transported with VTM" Viral transport media" for survival of virus alive until its arrival to laboratory. Of these samples, 71 case tested positivity for hMPV and RSV in previous study of 58 and 13 cases, respectively [12].

2.2 DNA extraction

Same samples were selected for DNA extraction to detect HBoV, using three types of kits: Geneius Micro gDNA Extraction kit Geneaid Biotech/ (USA), procedures were followed as described by the manufacturer's instructions.

2.3 Amplification by RT-PCR

All clinical samples tested with real-time PCR technique for detection of the designated viruses as a separated test of each, with specific primers and probes. One pair of specific primers are tested to reverse transcribe and amplify the human bocavirus (HBoV) highly conserved VP1/2 genes. Primers were highly specific for the VP1/2 capsid genes of the HBoV, the forward sequence primer was (5′-GCA CTT CTG TAT CAG ATG CCT T-'3), reverse primer (5′- CGT GGT ATG TAG GCG TGT AG-′3) and A DNA probe conjugated with 5' FAM fluorophore and 3' black hole quencher 1 (BHQ1) targeted this region (CCAGAGATGTTCACTCGCCG-MGB -′3) [13].

The master mix in use for the one step RT-PCR was the (QIAGEN) mix reagents. It was added to 3 µl DNA templates, 0.5 pmol conc. of each primer and 0.3 pmol conc. of the probe in 25 µl reaction mixture. The master mix reagents prepared according to the instruction kit. Amplification and detection were done with an Applied
Biosystem7500. Briefly, one cycle for 15 min at 94C°, followed by 45 cycles for 10 s at 95C° and 1 min at 60C°.

2.4 Statistical analysis

The Statistical Analysis System-SAS (2012) program was used to effect of difference factors in study parameters. All values were calculated according to the positive results as percentages (%). Differences between study groups and assays were analyzed by cross-tab and Pearson chi-square (X2) test. A value of $P<0.05$ was considered statistically significant.

III. Results and Discussion

Results showed 48/195 (24.62%) positive case for HBoV with threshold (Ct) values ranged from 15.25 to 38.7 (32.29 ± 6.5) (Fig.1). Furthermore, there is no statistical significant for viral present among suspected cases (X^2=0.621, P>0.05). No previous local study was performed to detect HBoV in Iraq, thus this value represent the first observation for further studies over it.

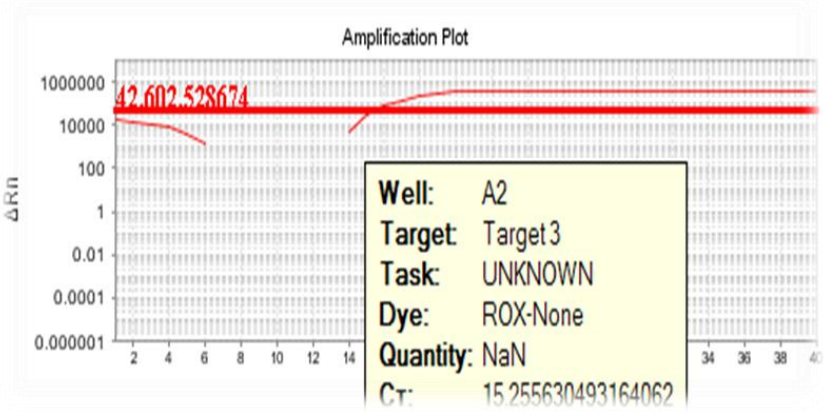

Figure 1: Real-Time PCR amplification plot of a positive Human Bocavirus sample, based on nucleoprotein gene primers and probe (ROX) dye of nasopharyngeal swabs for a patient with ILI symptom, this positive sample showed an amplification of 24.96 threshold cycle (Ct), all HBoV positive samples showed Ct amplification range of 15.25-38.7.

Generally HBoV1 DNA prevalence in young children with RTIs is about 10% but in some studies reported up to 33% [14]. The diversity of reported values may vary because of sampling techniques, study populations and the sensitivity of the detection assays [15]. In comparison of our results to other studies internationally and in the surrounding region, a clear and notable variation between viral infection cases among children suffering from ILI was observed. A RT-PCR-based study was conducted in Iran in 2013 to detect the prevalence of HBoV, showing 16/200 (8%) positive samples [16]. Another Iranian study conducted between 2012 and 2013 by using conventional PCR technique, showing 15 /140 (10.7 %) HBoV positive samples, these positive samples were negative for influenza A and B viruses [17]. In Kuwait, a study was conducted in 2014 by using RT-PCR technique, showed 14/285 (4.9 %) HBoV positive samples and 15/285 (5.3%) hMPV positives, while RSV showed 42/285 (14.7%) positives [18]. On the other hand, a study was conducted in Istanbul/ Turkey, between December 2013 and July 2014, a total of 1143 swabs from children with ILI were collected, showing 23(2%) HBoV positive, 36 (3.1 %) hMPV positive and 100 (8.7%) RSV positive samples. In a total overview on the whole viruses subjected under this study, we noticed a variation of single and mixed infections [19].

In current study, the mixed infections were recorded for HBoV with both viruses of the *Paramyxoviridae* family; positive mixed infection for HBoV with RSV showed 9 (4.62%) of all the positives; whereas 7(3.59%) positive samples were recorded for HBoV with hMPV, the remaining 32 (16.41%) of HBoV single infection and 147 (75.38%) samples showed negative results for all the mentioned above viruses (Fig.2). The statistical analysis for mixed infections of HBoVs with the two other viruses during this study has showed no significance with hMPV (x^2 = 1.3, P>0.05) and highly significance for HBoV with RSV(x^2 = 23.81, P > 0.001).

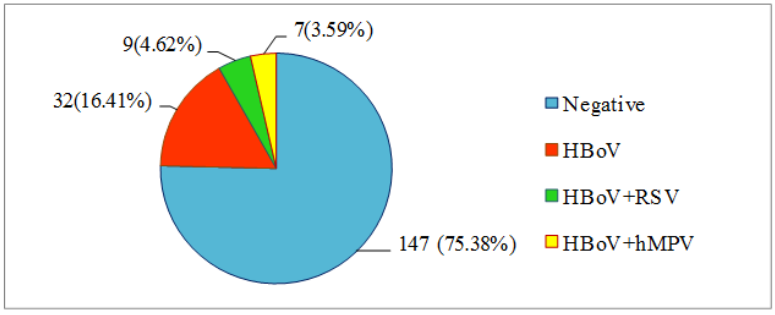

Figure 2: Molecular findings ratios for single and mixed infections.

The seasonal distributions of hMPV, RSV and HBoV have been shown to overlap; therefore potential for dual infection exists, There is however an increase in cases of HBoV co-infection with other respiratory tract viruses. This might be due to increased use of molecular techniques for diagnosis e.g. real time PCR. According to previously published researches, and the current study, HBoV showed its prevalence of co-infection with RSV circulation among Iraqi children with ILI, the prevalence rate obtained in our research was higher than previous studies, 9/195 (4.62%) versus 2/140 (1.4%) positives [17], the slight statistical increase in co-infections might be related to the increase in the number of collected samples in addition to technical related issues during the viral detection. In another regional study in turkey 2015 [19], HBoV was shown in 23/1143 samples, only 4/23 were positive for coinfection with RSV, another supportive indication for similar frequency of the HBoV-RSV infection rate. Reasons for that might be related to the synchronization in samples collection date in our studies and other studies in the region surrounding Iraq.

All the previously mentioned studies provided quite evidences that HBoV can be detected from nasopharyngeal samples of children affected by acute respiratory tract infection, with no differences between children HBoV alone or in co-infection state, and most importantly, this is study is documented as the first record for HBoV appearance in the local Iraqi community among children, in both mixed and single infections. Although very often HBoV1 infection is accompanied by co-infections with other respiratory viruses, however there are LRTI cases when HBoV1 is the only pathogen detected, indicating its possible role in etiology of the disease.

Although HBoV has been regarded as an infectious agent present, its pathogenic role in respiratory disease is still debatable. This virus is frequently detected in co-infection with other respiratory viruses of well-established pathogenic role [20, 21]. In this current study, high virus load were noted mainly in the absence of other testing respiratory viruses and suggesting a causative role for HBoV. However, low virus load were more detected in virus co-infection, and co-infection with RSV was more strongly affiliated with the patients with low virus load, suggesting that a low virus load in the nasopharynx seemed to be associated with long term shedding of HBoV DNA, unrelated to current illness. Actually, a recent study has suggested that asymptomatic viral

infection in infants is associated with low viral load [22], and that was supported by previous studies that showed that the high HBoV virus load played an important role in the severity of LRTI [23, 24]. Other investigators also found that high HBoV virus load led to more severe lower respiratory tract symptoms and longerhospitalization [25].

In our study, we found that age < 2 years and high virus load variable were associated with severe LRTI, while virus co-infection would not increase disease severity. Therefore the more severe lower respiratory tract symptom presented in high HBoV virus load patients may solely depend on HBoV virus load. Higher hMPV viral loads were significantly correlated with the course of illness and disease severity, irrespective of genotype [26].High levels of hMPV viral shedding lasts from 1 to 2 weeks after acute illness [27]. Despite the large number of mixed HBoV infections, our findings are still consistent with a potential etiologic role for HBoV in respiratory tract disease in young children.

According to the age of infected children, the cases were categorized into three groups, as show in the table 1 below, HBoV showed highly significant values (P<0.01) at 2 years and below age range, recording 68.75%. Positive cases for HBoV decreased dramatically with age increase (50- 180) months.

Table 1: Distribution of infection cases with HBoVs among different age groups/months

Virus	Age groups (month)	Total No.	Positive (+ve)	Negative (-ve)	Percentage of +ve in cases	Chi-square	P-value
HBoV	1-24	129	33	96	68.75	32.375**	0.001
	25-60	48	14	34	29.17		
	61-180	18	1	17	2.08		
Total No.		195	48	147	24.62		

The discovery of HBoV1 in children with respiratory disease rapidly prompted a large number of confirmatory studies assessing the presence of the virus in respiratory specimens. Since these are routinely taken from symptomatic individuals but only rarely from asymptomatic individuals, most studies have focused on HBoV1 prevalence among the former. A study conductedin 2011 in Brazil showed that 60 (13.2%) of 200 samples were HBoV-positive children, and the average age for that was 7.5 months, in a similar fashion to support our current study results [28].

According to the literature, the prevalence of HBoV infection among hospitalized children with respiratory tract infection ranges between 1.5-18.3% [29-31]. In our study, we found a prevalence of 24.61%, which is one of the highest, reported values so far. This result may be explained by the pre-selection of children with severe clinical manifestations. Rather than geographic differences in HBoV circulation, this discrepancy may be explained by differences observed in the cohort studied so far. Several reports included exclusively hospitalized children while others enrolled either hospitalized or outpatient children [32].

Other studies showed that HBoV has been detected in 0.6-4.3% of respiratory samples of children with RTI, usually together with other viruses [33, 34]. In contrast, a study conducted in Egypt showed different ratios of HBoV positive samples 2% (2/100 samples) at age groups 7-18 months [35]. This might be related to variations in molecular handling techniques, or the viral load was quite low to be detected by RT- PCR, or the sample collection was off-season.

As all of the respiratory viral agents were not ruled out in our hMPV RSV and HBoV positive children, we cannot conclude that these three viruses were the sole causative agents of all ARTI and LRTI in this study. Large prospective studies over longer periods of time can help to fully understand and to appreciate the clinical significance associated with this issue.

Meanwhile, the negative cases of suspected respiratory tract infection for subfamily *Pneumovirinae* (hMPV and RSV) and *Parvovirinae* (HBoV) may explain other causes of RTI that may be due to other viral infections. These could be several viruses are known to cause respiratory infections in humans [36]. Major pathogens that induce URIs are human rhinoviruses (HRVs), belongs to *Picornaviridae* [37]. Other common causative agents are adenovirus, parainfluenza virus, enterovirus (EV) from the family Picornaviridae and genus Paraechovirus and coronavirus [38, 39].

Moreover, HBoVs cases showed high significance among early winter beginnings (October-2015) with 6 (26.09%) positive samples, through mid-winter, while the highest number of positive cases were recorded on (February-2016) with a total of 18 (58.06%) positive HBoVs, and that was the peak of the recorded viral infections in this study (Table 2).

The activity of HBoV has been shown to be greatest in winter; the high prevalence of the virus during mid-winter (Feb-2016) makes a logical sense, in fluctuation with the rainy season, that leads to the drop- off in the Immune system status among children due to cold weather and in synchronization with the epidemic flu season and the high activity of other respiratory viruses. Infections with respiratory viruses exhibit distinct

seasonal patterns in most temperate regions. Most of these studies on these viruses have been conducted in the temperate regions; this is largely due to the availability of sensitive equipment such as real time PCR.

Table 2: Distribution of infection cases with HBoVs according to months of year during period study (2015-2016)

Month	Total No.	HBoV Positive (Percentage of +ve)
October	23	6(26.09)
November	21	1 (4.76)
December	30	4 (13.33)
January	31	7 (22.58)
February	31	18 (58.06)
March	28	6 (21.43)
April	26	2 (7.69)
May	5	4 (80)
Total No.	**195**	**48**
Chi-square	---	32.333 **
P-value	---	0.001

Out of 195 collected NPS, 119 cases were collected from Baghdad governorate, followed by Wasit (23), Babil(22), Karbalaa(10), Dhi Qar(8), Erbil(5), Al Najjaf(5), Diwanya(2) and Al-Muthana(1) governorate, all viruses showed significant values. Baghdad governorate showed with highest positive values for HBoV of 30 case, a highly significant value when compared to other governorates, the values were in accordance HBoV (X^2 94.042, P0.001) (Table 3).

Table 3: Distribution of infection cases with HBoVs among different Iraqi provinces

Governorates	Total No.	HBoV Positive (Percentage of +ve)
Baghdad	**119**	**30 (25.21)**
Babel	**22**	**4 (18.18)**
Karbala	**10**	**4 (40)**
Wasit	**23**	**6 (26.09)**
Diwaniah	**2**	**1 (50)**
Najaf	**5**	**2 (40)**
Muthanna	**1**	**1 (100)**
Arbil	**5**	**---**
Dhi-Qar	**8**	**---**
Total No.	**195**	**48**
Chi-square	**---**	**94.042 ****
P-value	**---**	**0.001**

It is difficult to determine the overall burden of disease caused by HBoV, hMPV and RSV due to seasonality and geographical variation. The highest prevalence for all the viruses in Baghdad is directly related to the age of the pediatric patients (mostly 4 years and below), these age ranges are considered to be more susceptible to viral infections (low immune system).

IV. Conclusion

In conclusion, Human bocavirus have been recognized and reported as the etiologic agents of respiratory tract infections predominantly in children with ILI, the distinguishing proof of new viruses will keep on occurring as molecular methodology tests turn out to be more contemporary. The prevalence rate for the HBoV single infection showed the viral load is higher than positive cases with hMPV and RSV as co-infection. No further study was ever done to detect HBoV in Iraq, by far, this is the first record, and the key reference for further studies over it.

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