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Human Bocavirus in Children with Respiratory Tract Infection: Molecular and Serological Detection

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ABSTRACT

Human bocavirus (HBoV) is a parvovirus identified mostly in children under the age of two who have a respiratory illness. The goal of this study was to use molecular and serological techniques to investigate the prevalence of HBoV as well as the clinical characteristics of Iraqi children with respiratory tract infections (RTIs). The study included 91 children ranging in age from 1–36 months. Nasopharyngeal/throat swab samples were collected, processed and tested for HBoV DNA detection by Polymerase Chain Reaction. In addition, Enzyme Linked Immunosorbent Assay (ELISA) was used to detect HBoV infection indirectly by measuring HBoV-IgM antibodies in serum. HBoV was found in 9.9% and 16.5% of samples, respectively, using the PCR and ELISA techniques. The ELISA test demonstrated a sensitivity, specificity, and positive predictive value of 90.2%, 77.8% and 97.4%, respectively, when compared to PCR. HBoV infection was associated with clinical symptoms such as vomiting and asthma ($P<0.05$). The PCR technique was effective in detecting persistent HBoV infections in hospitalised children; however, combining it with an ELISA test improved the accuracy of diagnosing acute HBoV infections (rather than bystander/passenger RTIs) by assessing specific IgM antibodies in serum.

KEYWORDS

Chest infection, HBoV, ELISA, PCR, under-five children, Iraq

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1. Introduction

Respiratory infections are the most common causes of death worldwide. Using sensitive viral detection methods, new viruses that could play a role in respiratory infection are being found at an increasing rate (Martin *et al.*, 2010). In 2005, Tobias Allander and his colleagues in Sweden detected an unknown virus belonging to Parvoviridae in children with respiratory tract diseases. The name "Human Bocavirus (HBoV)" was given to the virus (Zaghloul, 2011). HBoV is a single-stranded DNA virus that belongs to Parvoviridae (Parvovirinae subfamily) and Bocavirusgenus. It is tiny, icosahedral, and non-enveloped. It is categorised into four main species, HBoV1 through 4 (Bhat and Almajhdi, 2021).

The size of HBoV genome is around 5.3 kb and has three open reading frames (ORFs) that encode NS1 and NP1 non-structural and VP1 and VP2 structural proteins (Watanabe *et al.*, 2018). HBoV1 was originally discovered in children who had respiratory infections, but HBoV-2 to HBoV-4 are mostly detected in feces and only rarely in the respiratory tract (Kantola *et al.*, 2011; Broccoloa *et al.*, 2015; Watanabe *et al.*, 2018). However, because HBoV is frequently associated with other agents and Koch's modified postulates are inapplicable, there is debate about HBoV's true role as a respiratory and gastrointestinal pathogen (Chuang *et al.*, 2011, Watanabe *et al.*, 2018; Bhat and Almajhdi, 2021).

The virus has been found in children with RTIs around the world, showing that it is prevalent (Sloots *et al.*, 2006; Chuang *et al.*, 2011). The total prevalence of HBoV is reported to be 6.3%, with an average ranging from 1.0% to 56.8% worldwide (Guido *et al.*, 2016). Pneumonia, bronchitis, and bronchiolitis are the most prevalent HBoV symptoms (Lau *et al.*, 2007). There have been numerous life-threatening reported cases of respiratory HBoV1 infections (Ziemele *et al.*, 2019).

Traditional PCR, real-time PCR, ELISA, recombinant capsid proteins (VP2) or virus-like particle (VLP) based immunoassay are currently used to detect HBoV.In addition, Sequence-Independent, Single-Primer Amplification (SISPA) techniques in combination with Next Generation Sequencing (NGS) will be applied in future for rapid and simultaneous detection of pathogen sequences (Hao*et al.*, 2015; Guido*et al.*, 2016). Until now, there have been no cell lines and animal models available for Bocavirus isolation, therefore, the mechanisms of latency, persistence, and reinfection are currently unknown (Bhat and Almajhdi, 2021).

HBoV has been detected in nasal, blood, saliva, feces, urine and environmental samples (i.e. river water and sewage), which has raised worries about its existence in transfusion therapy (Zaghloul, 2011; Guido *et al.*, 2016; Mohammad *et al.*, 2019). The goal of this study was to see if there were any associations between HBoV detection at the molecular and serological levels in Iraqi children with respiratory infections and clinical characteristics.

2. Material and Methods

2.1. Specimen Collection:

Samples were collected from 91 children under the age of five with respiratory infections who were admitted to paediatric hospitals (Al-Imammain Al-Kadhimain Teaching Hospital, Al Kadhimiya Pediatric Hospital, and Central Teaching Hospital of Pediatrics)in Baghdad, Iraq, from January to April 2017. This study was authorised by the Institute Review Board at Al-Nahrain College of Medicine, and the children's parents or guardians gave their informed consent. An attending physician examined all the patients. Three-millilitre universal transport media with nasopharyngeal/throat swabs (Cat.#: 80346C & 80503CS, Copan Diagnostic, Italy) were used. The samples were kept at -80°C. Furthermore, leftover blood from the chemistry laboratory was collected and serum was separated at 3000 rpm for 20 min and stored at -20°C until it was used.

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2.2. DNA Extraction:

Following the manufacturer's instructions, DNA samples were extracted using DNA/RNA prep kits from Sacace Biotec. The concentration and purity of DNA were measured using BioDrop. The extract was kept at -20°C until it was used.

2.3. Polymerase Chain Reaction (PCR):

The lyophilised primers 188F (5′-GAGCTCTGTAAGTACTATTAC-3′) and 542R (5′-CTCTGTGTTGACTGAATACAG-3′) (Kaplan *et al.*, 2006) that target the non-structural protein-1 gene (NP-1, which is more conserved than the VP1/2 gene, *y*ielding a 354 bp amplicon) were generated per the manufacturer's directions (Alpha DNA, Canada). The PCR reaction mixture included the following: 12.5 µl of GoTaq (R) Green Master Mix (Promega, USA), 2 µl of each forward and reverse primer, 3 µl of DNA extract, and nuclease-free water (Promega, USA), bringing the total volume to 25 µl. In PCR Thermal Cycler (Eppendorf, Germany), the following program was utilised: initial denaturation at 95°C for 5 min, then 35 cycles of denaturation, annealing and extension at 94°C for 1 min, 59°C for 1 min, and 72°C for 2 min, respectively, followed by a final extension at 72°C for 10 min. To confirm amplification, agarose electrophoresis on gel (1%) (Promega, USA) with ethidium bromide (0.5 mg/ml) (Promega, USA) in 1xTBE buffer (Promega, USA) using a 100-bp DNA marker (Promega, USA) was done. The PCR products consisted of one band of 354 bp. To monitor assay performance, positive (HBoV-positive samples) and negative (nuclease-free water) controls were included in all runs (Figure 1).

2.4. DNA Sequencing:

For confirmation, one HBoV amplicon was chosen for Sanger sequencing. The sequencing done by Macrogen Inc. The observed DNA sequences were then compared to the retrieved HBoV DNA sequences (GenBank acc. MF314144.1) on the NCBI. The local isolate sequences were deposited at GenBank under the acc.MH591416 (HBoV-1/Baghdad-82/Iraq/2018).

2.5. Serology:

Ninety-one serum samples were analysed, according to the manufacturer's directions, for HBoV IgM antibodies using ELISA (Glory Science Co., Ltd, China). Antibodies specific to Bocavirus-IgM were coated on the Sandwich-ELISA microtiter plate. To prepare the sample, serum was diluted 5-fold with 40µl of sample diluents from the kit and 10µl of the serum samples to sample wells and, thereafter, incubated for 30 min at 37°C. After that, the wells were washed five times and a Horseradish Peroxidase (HRP), labelled anti-Bocavirus-IgM, was added to each well (except the blank well) and incubated for 30 min at 37°C to bind them to the HBoV IgM. Free components were washed five times. All wells had chromogen A and B added to them, and they were incubated in the dark for 15 min at 37°C. After the addition of the stop solution, the optical density (OD) of each well was determined at a wavelength of 450 nm and interpreted as positive or negative when compared to the cut-off value.

2.6. Statistical Analysis:

Based on PCR as the gold standard method, the validity and predictability of ELISA (sensitivity, specificity, positive predictive value (PPV) and negative predictive values (NPV)) were evaluated by Chi-Square (χ^2). Also, χ^2tests were used to determine the association between the presence of HBoV infection, clinical data and demographic data. Statistical significance was defined as a *P* value of < 0.05.

3. Result

3.1. Frequency of HBoV:

This cross-sectional study included 91 children. Their mean age (6.87 ± 6.38 SD) ranged from 1–36 months, with 38 (41.8%) females and 53 (58.2%) males. A combination of HBoV-DNA detection by PCR and/or HBoV-IgM detection by ELISA resulted in a total HBoV detection in 17 (18.7%) of 91 patients (Table 1). Only nine of 91 patients (9.9%) tested positive for HBoV-DNA, and only two of them were not identified by ELISA. However, 15 of 91 patients (16.5%) tested positive for anti HBoV IgM and seven of them were confirmed by PCR. Based on PCR, the sensitivity, specificity, PPV and NPV were calculated to compare PCR and ELISA. According to the findings of this study, the sensitivity of ELISA was 90.2% and the specificity was 77.8%, with a likelihood ratio of 19.49 (Table 2).

3.2. Demographic Data and Clinical Characteristics:

The age distribution revealed that 88.9% of HBoV-DNA-positive samples and 86.7% of HBoV-IgM-positive samples came from children aged 1 to 12 months (*P*=0.007) and (*P*=0.075), respectively. Fever, cough, dyspnea, cyanosis, vomiting, pneumonia, bronchiolitis, asthma, weight loss, pertussis and wheezing are common clinical symptoms in this age group (Table 1).

There was no statistical significance between HBoV DNA and/or IgM status and the sex or mean age (*P*>0.05). Furthermore, although not statistically significant, HBoV infections were found in 15.8% of children with pneumonia and 22.2% of children with bronchiolitis. Clinical symptoms of HBoV infection such as fever, cough, dyspnea, cyanosis and weight loss were not significant (*P*>0.05). However, clinical symptoms such as vomiting (*P*=0.028) and asthma (*P*=0.002) were statistically significant (*P*<0.05) (Table 3).

Table (1): Study population and HBoV infection in comparison with age groups.

Characteristics		Age Group			*P*-value
		1–12 m No. (%)	13–24 m No. (%)	25–36 m No. (%)	
Demographics					
Age in months (mean 6.87 ± 6.38 SD)		82 (90.1)	8 (8.8)	1 (1.1)	0.000*
Sex	Female (n=38, 41.8%)	1 (2.6)	3 (7.9)	34 (89.5)	
	Male (n=53,58.2%)	0 (0.0)	5 (9.4)	48 (90.6)	0.483
Clinical Signs and Symptoms					
Fever (n=77)		70 (90.9)	6 (7.8)	1 (1.3)	0.675
Cough (n=72)		64 (88.9)	7 (9.7)	1 (1.4)	0.719
Dyspnea (n=44)		42 (95.5)	2 (4.5)	0 (0.0)	0.228
Cyanosis (n=5)		5 (100)	0 (0.0)	0 (0.0)	0.748
Vomiting (n=12)		9 (75)	2 (16.7)	1 (8.3)	0.019*
Pneumonia (n=38)		35 (92.1)	3 (7.9)	0 (0.0)	0.668
Bronchiolitis (n=9)		9 (100)	0 (0.0)	0 (0.0)	0.578
Asthma (n=6)		5 (83.3)	1 (17.6)	0 (0.0)	0.757
Weight loss (n=13)		12 (92.3)	1 (7.7)	0 (0.0)	0.907
Pertussis (n=5)		5 (100)	0 (0.0)	0 (0.0)	0.748
Wheezing (n=17)		16 (94.1)	1 (5.9)	0 (0.0)	0.791
Viral Infection					
HBoV-DNA (n=9, 9.9%)	+ve	8 (88.9)	0 (0.0)	1 (11.1)	0.007*
	-ve	74 (90.2)	8 (9.8)	0 (0.0)	
HBoV-IgM-Ab (n=15, 16.5%)	+ve	13 (86.7)	1 (6.7)	1 (6.7)	0.075
	-ve	69 (90.8)	7 (9.2)	0 (0.0)	
HBoV DNA&/or IgM (n=17, 18.7%)	+ve	15 (88.2)	1 (5.9)	1 (5.9)	0.102
	-ve	67 (90.5)	7 (9.5)	0 (0.0)	

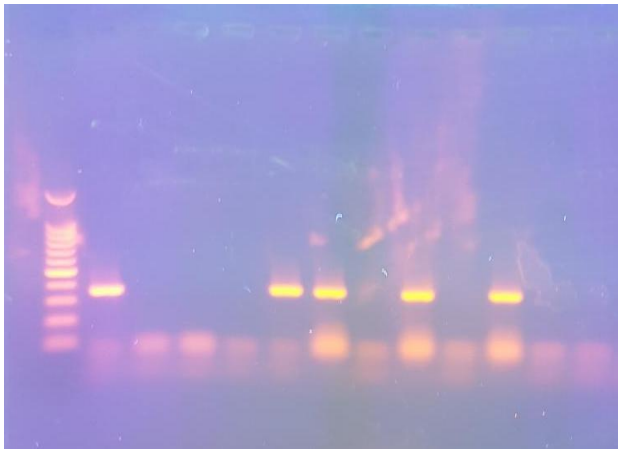
Table (2): ELISA sensitivity and specificity confirmed by PCR.

Category		PCR (n =91)		Total No. (%)
		HBoV-DNA +veNo. (%)	HBoV-DNA -ve No. (%)	
ELISA (n =91)	HBoV-IgM +ve	7 (77.8)	8(9.8)	15(16.5)
	HBoV-IgM -ve	2 (22.2)	74(90.2)	76(83.5)
	Total No. (%)	9 (9.9)	82 (90.1)	91 (100)
Statistics		P<0.001		
Sensitivity		90.2 %		
Specificity		77.8 %		
PPV		97.4 %		
NPV		47.7 %		
Likelihood Ratio (LR)		19.499		

Table (3): Demographics, clinical signs and symptoms with regard to HBoV status.

Characteristics		HBoV DNA &/or IgM		*P*-value
		+ve No. (%)	-ve No. (%)	
Demographics				
Age (mean ± SD) months		17 (18.68) (6.64±9.35)	74 (81.32) (6.91±5.56)	0.875
Sex	Female (n=38)	8 (21.1)	30 (78.9)	0.623
	Male (n=53)	9 (17)	44 (83)	
Clinical signs and symptoms				
Fever (n=77)		14 (18.2)	63 (81.8)	0.774
Cough (n=72)		15 (20.8)	57 (79.2)	0.305
Dyspnea (n=44)		8 (18.2)	36 (81.8)	0.906
Cyanosis (n=5)		0 (0.0)	5 (100)	0.270
Vomiting (n=12)		5 (41.7)	7 (58.3)	0.028*
Pneumonia (n=38)		6 (15.8)	32 (84.2)	0.549
Bronchiolitis (n=9)		2 (22.2)	7 (77.8)	0.774
Asthma (n=6)		4 (66.7)	2 (33.3)	0.002*
Weight loss (n=13)		3 (23.1)	10 (76.9)	0.661
Pertussis (n=5)		1 (20.0)	4 (80)	0.938
Wheezing (n=17)		1 (5.9)	16 (94.1)	0.133

Figure 1: HBoV amplicon electrophoresis. Lane 1 represents a DNA marker, lane 2 represents a positive control and lane 3 represents a negative control. Negative samples in lanes 4, 5, 8, 10, 12 and 13, and positive samples in lanes 6, 7, 9 and 11.



3.3. HBoV1 DNA Sequencing:

HBoV genomic sequences were highlighted in (Table 4). The amplified sequence was extended from 2340 to 2693 of the NCBI reference DNA sequence (GenBank acc. no. MF314144.1). The grey regions refer to forward and reverse primers. The alignment of the sequenced sample showed the absence of any mutation in the analysed specimen when compared to the referring HBoV sequences (Figure 2). Local isolate sequences were deposited at GenBank (acc. MH591416).

The NP1 amino acid sequence that was included within the 354 bp amplicon is:

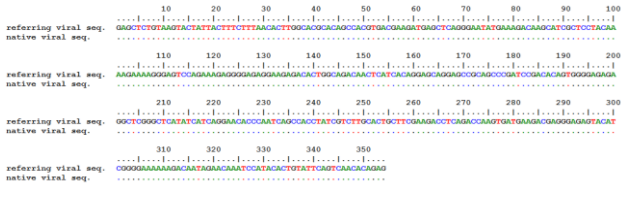
[MSSGNMKDKHRSYKRKGSPERGERKRHWQTTHHRSRSRS
PIRHSGERGSGSYHQEHPISHLSSCTASKTSDQVMKTRESTSG
KKDNRTNPYTVFSQHR]

Table (4): The PCR amplicon within the HBoV genomic locus.

Amplicon	Referring locus sequences (5′–3′)*	Length
HBoV	GAGCTCTGTAAGTACTATTACTTTCTTTAACACTTGGCACGCACAGCCACGTGACGAAGATG AGCTCAGGGAATATGAAAGACAAGCATCGCTCCTACAAAAGAAAAGGGAGTCCAGAAAGA GGGGAGAGGAAGAGACACTGGCAGACAACTCATCACAGGAGCAGGAGCCGCAGCCCGAT CCGACACAGTGGGGAGAGAGGCTCGGGCTCATATCATCAGGAACACCCAATCAGCCACCT ATCGTCTTGCACTGCTTCGAAGACCTCAGACCAAGTGATGAAGACGAGGGAGAGTACATCGGGG AAAAAAGACAATAGAACAAATCCATACACTGTATTCAGTCAACACAGAG	354 bp

*The reverse primer was placed in a reverse complement mode.

Figure 2. DNA sequences alignment of the observed local viral sequences with its corresponding reference sequences of the 354 bp amplicon within the HBoV-DNA sequences. The symbol "ref" refers to the NCBI referring sequence.



4. Discussion

In this study, only nine cases (9.9%) of children with respiratory illnesses tested positive for HBoV-DNA infection (Table 1). This result was predicted by considering the prevalence of HBoV in respiratory samples. The prevalence of HBoV infection varies from 2.7–19%, with the majority occurring in children under the age of two (Lindner and Modrow, 2008; Ziemele *et al.*, 2019). In addition, HBoV DNA was 56.8%, 19%, 18.3%, 7.6%, 5%; 2.3% and 1.9%, in Egypt, Finland, Jordan, Iran, Taiwan, Turkey and Kuwait, respectively (Kaplan *et al.*, 2006; Allander *et al.*, 2007; Chuang *et al.*, 2011; Guido *et al.*, 2016), depending on the study design, laboratory technology, patient age and symptoms (upper or lower RTI) (Kantola *et al.*, 2008; Chuang *et al.*, 2011; Gu et *al.*, 2017). However, the low prevalence in our findings may be due to the small samples size used, which was limited to one region of Iraq and one winter season. Although HBoV infections have been found primarily in winter (January and February) (Guido *et al.*, 2016; Mohammad *et al.*, 2019), a rise in infections has been observed in the spring and summer (Lindner and Modrow, 2008; Calvo *et al.*, 2010).

When molecular and serological techniques were compared (Table 2), eight (9.8%) patients had HBoV-IgM-positive but PCR-negative results. This discrepancy could be due to blood being taken after viral nucleic acid was eliminated from the respiratory tract, or due to hospitalised patients requiring more time before testing (Al-Shuwaikh *et al.*, 2018). Furthermore, depending on assay sensitivity, the samples could have low HBoV loads (Allander *et al.*, 2007). Serology antigens are based on HBoV (Broccoloa *et al.*, 2015), and several studies have shown that despite there being no cross reactivity between the two human parvovirus's antibodies (i.e. HBoV and Parvovirus B19 antibodies) (Endo *et al.*, 2007; Kantola *et al.*, 2008; Malecki *et al.*, 2011), HBoV2–4 co-circulation may influence HBoV serological tests (Kantola *et al.*, 2011; Hao*et al.*, 2015; Ziemele *et al.*, 2019). The unavailability of monospecific HBoV antibodies is one of the key obstacles in understanding the epidemiology of HBoV (Hustedt *et al.*, 2012). Serology, on the other hand, is critical because viremia can only be detected during active infection (Broccoloa *et al.*, 2015). Lindner *et al.* (2008) stated that their ELISA had high sensitivity and specificity with a PPV of 97% in wheezing children. In agreement with our findings, Zaghloul (2011) mentioned that PCR and ELISA were used to detect the presence of HBoV and both tests were extremely sensitive and specific. According to one study, serologic analysis or serum PCR are required for an accurate HBoV diagnosis, whereas PCR of nasopharyngeal aspirates alone is insufficient (Söderlund-Venermo *et al.*, 2009).

HBoV infections in the airways are systemic and cause humoral immune responses (Allander *et al.*, 2007; Kantola *et al.*, 2008; Ziemele *et al.*, 2019). Humans are infected with HBoV2–4 less frequently than HBoV1 and have weaker B-cell responses (Kantola *et al.*, 2011). IgG seropositivity increased with age (Endo *et al.*, 2007; Malecki *et al.*, 2011), reaching up to 95% in adults (Moesker *et al.*, 2015). As a result, discrepancies in results may also be related to variances in the age groups studied in different studies (Hao *et al.*, 2015). Our findings show that two children had HBoV-positive PCR results but no HBoV-IgM Ab (Table 2), this could be explained by the fact that a low viral load does not always indicate acute primary infection, which is consistent with previous findings (Söderlund-Venermo *et al.*, 2009). Furthermore, HBoV is a common virus that can cause persistent infections (without viremia) in the respiratory tract mucosa (Broccoloa *et al.*, 2015). HBoV is also commonly transmitted among children in day care or within families, which makes it easy for airways to be contaminated (Kantola *et al.*, 2008). As a result, interpreting PCR results in a clinical setting can be as difficult as interpreting serological testing.

According to the findings of this study, there is no statistically significant corelation between HBoV DNA and/or IgM status and the sex or mean age of the patient, which is similar to the findings of Mohammad *et al.* (2019) (Table 3). The majority of the children aged (1–12m) had HBoV-DNA-positive (88.9%, *P*=0.007) and HBoV-IgM-positive results (86.7%, *P*=0.075), but there was no clear link between HBoV infection and clinical symptoms except for those of vomiting (*P*=0.019) (Table 1) and asthma (*P*=0.002) (Table 3). The fact that many viruses exhibit clinical symptoms that are similar (Calvo *et al.*, 2010; Malecki *et al.*, 2011; Ziemele *et al.*, 2019) makes it unclear whether the symptoms were caused by the other viruses**.** Our findings revealed that four out of six (66.7%) asthmatic children were infected with HBoV (a significant corelation) (Table 3). However, viral infections in airways are present in a wide range of asthma patients during exacerbations (Costa *et al.*, 2014), with rhinovirus (RhV), respiratory syncytial virus (RSV) and HBoV being the most commonly found (Mandelcwajg *et al.*, 2010). Also, a significant relation was found between HBoV infection and vomiting. This may be due to respiratory viruses in infants frequently being associated with gastrointestinal manifestations. In addition, according to one study, up to 18% of hospitalised children with pneumonia tested HBoV positive (Kaplan *et al.*, 2006). HBoV has been shown in other studies to infect the lower respiratory tract, including the bronchioles (Söderlund-Venermo *et al.*, 2009; Calvo *et al.*, 2015; Guido *et al.*, 2016; Mohammad *et al.*, 2019). According to several studies, 6.6% and 40% of hospitalised HBoV- infected patients, require an intensive care unit stay and oxygen therapy, respectively (Arnold *et al.*, 2006; Chow *et al.*, 2008; Chow *et al.*, 2009).

Our results showed that one out five(20%)of patients had HBoV co-infection with Pertussis (Table 3). Patients with HBoV have a significant rate (83%) of co-infection (Bhat and Almajhdi, 2021). Infants and young children have immature immune systems (Ali *et al.*, 2019), therefore, infections with more than six pathogens in a single patient requires hospitalisation. HBoV tends to be associated with other respiratory viruses and bacteria, e.g. RSV, human RhV, adenovirus, metapneumovirus and *Streptococcus* spp (Lindner and Modrow, 2008, Malecki *et al.*, 2011; Mohammad *et al,* 2019). However, Moesker*et al.* (2015) found that in children, a single HBoV infection could induce severe acute respiratory infection (SARI) even in the absence of other viral and bacterial infections. In addition, a high viral load (>10^4 copies/ml) has been linked to more severe clinical symptoms and extended hospitalisation times (Broccoloa *et al.*, 2015; Guido *et al.*, 2016). However, this study cannot exclude other viral and bacterial co-infections. Importantly, the samples had previously been tested for RSV-RNA, which was found in 44% of the cases (data not shown). Rasheed *et al.* (2019) reported that 71% of children (<5 years) with lower RTI had such a viral infection, whether solitary or mixed, and two out of six HBoV-positive children had co-infection (HBoV and RhV).

Mutation and recombination events cause genetic variations in HBoVs (Broccoloa *et al.*, 2015). Based on the NP1 gene, sequenced HBoV local isolates(HBOV/Baghdad/82/2017) (GenBank acc. MH591416) have been identified with high sequence homology with the reference isolate (HBoV/ACRI_0111/USA/2016) (GenBank acc. MF314144.1) in Table 4 and Figure 2, indicating that HBoV is distributed worldwide. The non-structural genes were present, conserved regions of the viral genome. Further studies on the genes encoding the capsid proteins are needed since they showed the most variation, especially at its 3`end (Lindner and Modrow, 2008; Mohammad *et al.*, 2019).

5. Conclusion

This study has demonstrated the prevalence of HBoV in hospitalised children with RTIs in Baghdad, Iraq. The occurrence of HBoV was not statistically associated with clinical manifestations such as fever, cough, dyspnea, cyanosis, weight loss, pneumonia and bronchiolitis. However, a significant association was found in HBoV infection with vomiting and asthma. The PCR technique was more sensitive and specific than ELISA for the diagnosis of HBoV. ELISA, however, was a reliable serologic technique for detecting acute HBoV infections by measuring IgM antibodies in serum.

Biography

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