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Detection of Human Bocavirus in Egyptian Children Suffering from Acute Lower Respiratory Tract Infection

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A B S T R A C T

Human bocavirus (HBoV) was first identified as parvovirus in 2005 in nasopharyngeal aspirates of children with lower respiratory tract infection**.** The objectives of this study were to determine HBoV as a cause of acute lower respiratory tract infection(ALRTI) in children by investigating the presence of HBoV DNA, to evaluate diagnostic efficiency of HBov IgM assay in the serum of the studied patients and to assess the impact of the viral load on the clinical manifestations of children with positive HBoV DNA samples. We investigated 123-nasopharyngeal aspirate samples of hospitalized children with ALRTI by Real-time PCR for detection of HBoV DNA and estimating its viral load. Estimation of immunoglobulin M (IgM) by ELISA in patient's serum samples was another method used for diagnosis of HBoV infection. There were 19 patients with positive nasopharyngeal aspirate samples by PCR among them there were 16 serum samples positive for HBoV IgM by ELISA (sensitivity was 84.2%). Longer hospital stay and severe ALRTI signs were detected in children samples with high viral load (16 samples).

Introduction

Acute respiratory tract infection is a leading cause of significant morbidity worldwide. It is one of the most important causes of mortality in infants and young children and disability-adjusted life years lost in developing countries (WHO 2009). Although some viruses, such as influenza virus, respiratory syncytial virus and human adenovirus are responsible for most acute respiratory tract infection, however, some etiological causes remain unknown (Pavia 2011).

Human bocavirus 1 (HBoV-1) was first identified in 2005and defined as DNA virus belongs to the Parvoviridae family that has been frequently detected in young children experiencing acute respiratory tract illness (Allander *et al.*, 2005). Three other bocaviruses, HBoV2, 3, and 4, were detected in human Feces (Kapoor *et al.*, 2009), and HBoV2 has been associated with acute gastroenteritis (Arthur *et al.*, 2009). The role of HBoV as the single causative agent for respiratory tract infections remains unclear; few studies were carried out in Egypt to estimate the role of this virus in ALRI in children. The infection by HBoV1 can be diagnosed by many techniques that include serology and PCR amplification of viral nucleic acid, which is the most common

technique used for the detection of HBoV1 in respiratory samples (Bruning *et al.,* 2016). The aim of this work was to investigate the role of HBoV as a cause of acute lower respiratory tract infection (ALRTI) in children. We used real- time PCR for detection of HBoV DNA in nasopharyngeal aspirate (NPA) samples and we also investigated the diagnostic efficiency of HBoV IgM detection in patient's serum. Investigation of the relation between different viral load and the clinical characteristics of the children with ALRTI was another goal of this study.

Materials and Methods

A total of 123 children less than 60 months (5years) of age with a clinical and radiological diagnosis of ALRTI in Children's Hospital of Zagazig University from May 2015 to April 2016 were enrolled in this study. All the investigations were performed following the relevant guidelines and regulations of Zagazig University. The methods were carried out in accordance with the approved guidelines. The parents of all study participants gave written informed consent before study enrollment.

The patients were suffered from acute lower respiratory tract infection with onset during the previous seven days requiring hospitalization that included either history of fever and/or current fever ≥ 38°C or current hypothermia < 35.5°C and at least one of abnormal breath sounds, tachypnea (according to age), chest wheeze, cough, or respiratory distress (nasal flaring, chest in drawing, grunting).Clinical implication of HBov infection was assessed by clinical symptoms and length of hospital stay.

Specimens

Approximately 2 ml nasopharyngeal aspirates (NPA) samples were obtained from all patients within 24 hours of admission. For HBoV-IgM detection by ELISA, 3ml of blood was withdrawn from patients and the serum samples were separated.

For diagnosis of bacterial infection, NPA specimens were inoculated on MacConkey agar, blood agar and chocolate agar plates that incubated in aerobic, CO_2 condition at 37°C for 24-48 hours. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) was used to identify bacterial isolates. Another part of the specimens was stored in aliquots at -80°C for PCR. Serum samples were separated and stored at - 20°C for HBoV-IgM antibodies detection.

Real-time PCR

The total nucleic acids were extracted from the NPA samples using QIAamp MinElute virus spin DNA/RNA extraction kit (Qiagen, USA) following the manufacturer's instructions. Briefly, 200 µL of sample was extracted and the nucleic acids were eluted in 20-150 µL of eluted buffer and stored at -80°C.

The presence of human bocavirus genomes were detected from the extracted nucleic acid by real-time quantitative polymerase chain reaction (PCR) with commercial HBoV real-time kit (The Primer design™ genesig® Human bocavirus viral protein gene advanced kit) according to the manufacturer's instructions using Stratagene Mx3005P qPCR instrument (Agilent Technologies, USA).

A total reaction mix for each DNA sample prepared was 20 µl consisting of 10 µl of oasig™ lyophilised master mix, 1µl of HBoV primer/probe mix, 1µl of Internal extraction control primer/probe mix, 3µl RNAse/DNAse free water and 5 µl of extracted DNA template. The PCR amplification thermal cycling conditions were as the following: 15

min at 37°C, enzyme activation step of 2 min at 95°C and 50 cycles consist of: denaturation step was 10 sec at 95°C and annealing/elongation step of 1 min at 60°C. To avoid cross-contamination of the reaction, negative control (5µl of RNAse/DNAse free water instead to DNA template) and 6 serial dilutions of the positive control that had been provided in the kit, were included in each PCR assay. The fluorescent signal from the FAM channel was measured against the signal from VIC channels, in order to normalize the non-PCR-related fluorescence fluctuations between samples. A standard curve of amplification was produced using serial dilutions of a positive control; the quantitative calculation of the viral loads from the cycle threshold (Ct) was performed according to the standard curve (copies/ml).

Serology

HBoV-IgM antibody levels were determined by enzyme-linked immunosorbent assay (ELISA) supplied by NOVA (Beijing, China) in all patients' serum samples according to the manufacturer's instructions. The absorbance was read at 450 nm. The average of negative and positive controls results were ≤0.10and ≥1.00 respectively, the calculating critical (cut off) was the average of negative control + 0.15

Statistical analysis

The data was analyzed using SPSS statistical package software computer program version 17 (SPSS Inc., Chicago, IL). Shapiro – Wilk test was used to verify the normality of distribution of continuous variables. Data were expressed as a number of percentages, mean or median as appropriate. Categorical data were analyzed using the Chi- square test and Fisher's exact and numerical data are analyzed by Mann-Whitney U Tests. Receiver operating characteristic (ROC) curve was used to calculate cut off of high viral load. Statistical significance for all the tests was set at a P-value of less than 0.05 (< 0.05). Sensitivity, specificity, PPV and NPV was calculated to evaluate the diagnostic efficiency of HBoV detection.

Results and Discussion

The mean age of the children included in this study was 37months. The clinical finding and epidemiological characteristics of patients with HBoV DNA positive samples by real-time are illustrated in table 1. The detection of HBoV was in its peak by age range from 6 to 12 months (Figure 1) and its incidence was highest in winter in comparison to other seasons (Figure 2).

This study showed that among 123 NPA samples obtained from children with ALRI there were 19 positive results for HBoV DNA by real-time PCR technique (15.4%). There were 16 positive serum samples for HBoV IgM by ELISA (13%), all these samples also belonged to patients who had positive PCR results (16/19) (84.2%), and there were three patients with HBoV DNA positive PCR results and negative HBoV IgM. The sensitivity and specificity, PPV and NPV of ELISA are illustrated in table 2.

According to the positive results of HBoV DNA by real-time PCR we divided the results into high and low viral load groups, 1.0×10^4copies/mL was the cutoff value that was calculated using ROC curve. There were 13 samples with high viral load and 6 samples with low viral load values. There was a significant association between high viral load estimated by real-time PCR and increase the length of hospital stay, a presence of dyspnea, wheeze and tachycardia, while there was no significant association between the values of viral load and the presence of other clinical signs (Table 3). There was bacterial co-infection in three HBoV positive NPA samples that were *Streptococcus pneumoniae*,

S. aureus and *E. coli*, all these samples were accompanied by a low viral load.

Human bocavirus has been described as an etiological agent of respiratory virus infections since 2005; however, there was a lack of information about the role of this pathogen in the etiology of acute lower respiratory infections among the Egyptian children. We determined the incidence of HBoV by real-time PCR and serology in children suffering from ALRI and investigated the clinical significance of different viral load of this virus.

Our results showed that the detection rate of HBoV DNA from NPA samples of children with ALRI was 15.4%, that was in agreement with the results reported by Symekher *et al.,* (2013) that was(16.8%)also it was in agreement with other studies which have had a detection range of between 1.5% - 19% (Allander *et al.,* 2007,Silva2010,Al-Rousan2011) and with another study by Dunn and Miller (2014)that reported, HBoV can be detected to in 1.6 to 21.5% of children with symptoms of respiratory tract infection.

However, our results were lower than the results of two previous studies in Egypt by Abdel-Moneim *et al.,* (2016), Zaghloul (2011)who reported that HBoV was found in 56.8% and 24.5% respectively in the examined children in their studies, also our result was lower than that reported by Denge *et al.,* (2012) that was 22% and higher than those reported by Weissbrich *et al.,* (2006) and Neske *et al.,* (2007) who had found HBoV DNA by PCR techniques in NPA samples were 10.3%, 12% respectively. This variation may be due to different age groups of patients, different methods of detection of the virus.

In the current study, the detection rate of HBoV infection was at its peak between the ages of 6 and 12 months (57.9%). Our results was in agreement with Jiang *et al.,* (2016) who reported that the infections almost completely confine to infants and young children also in agreement with most of the previous studies by Allender *et al.,* (2005), Choi *et al.,* (2006), Chow *et al.,* (2009) and Abdel-Moneim *et al.,* (2013). However, some authors reported that HBoV is also a frequent virus in adults with respiratory disease Guido *et al.,* (2012) and Ghietto *et al.,* (2012).

In this study all HBoV positive cases occurred during late fall and winter, this finding is consistent with same findings by other authors Allander *et al.,* (2005), (Weissbrich *et al.,* (2006), Chow *et al.,* (2008) and Martin *et al.,* (2010).

Real-time PCR has been used as an indicator of the degree of active viral infection, interactions between the virus and the host, and the role of viral reactivation or persistence in the progression of disease (Mackay 2004). Our results showed that there were significant statistical differences between patients with high-viral-load and low-viral-load groups as the tachypnea, dyspnea and wheeze were associated with the higher viral load. These results were in agreement with Kim *et al.,* (2011). We found that the more severe lower respiratory tract symptoms in our patients were associated with high HBoV virus load that was in agreement with Jiang *et al.,* (2016).

Also, we found that the longer hospitalization period was accompanied with high HBoV viral load patients and that was in agreement with Deng *et al.,* (2012).These results suggest that HBoV is a significant pathogen in young children with ALRI that was underestimated in our region. We detected three NPA samples with HBoV positive PCR results that were accompanied by bacterial co-infection and were associated with low virus load that result was in agreement with Jiang *et al.,* (2016) who reported that co-infection was

more frequently found among patients with low virus load than those with high virus load suggesting HBoV as causative agent of ALRI, this results were in agreement with the studies of Brieu *et al.*, (2008) and Kaida *et al.*, (2010).

We estimated HBoV IgM as another diagnostic tool for diagnosis of HBoV causing ALRI and we found that there were13/ 123 (16%) positive cases by ELISA, these results were in agreement with previous results of IgM detection in children with HBoV in the studies by Zaghloul (2011) and Söderlund-Venermo *et al.*, (2009) that were 18%, 19% respectively.

There were16 positive IgM serum samples among 19HBoV DNA-positive patients (84.2%) diagnosed by real-time PCR, which is highly sensitive technique. This study result was in agreement with that of Zaghloul (2011). However, our results were higher than that of Lindner *et al.*, (2008) who detected IgM against HBoV by ELISA in (42%) of sera samples obtained from HBoV DNA-positive children this difference may be due to different PCR technique used.

There was significant association between high viral load and positive serology that probably indicate active HBoV infection; there were 13IgM-positive serum samples of patients who had high viral load among 16 IgM-positive serums. The three negative serological results had low viral load, this result was in agreement with Allander *et al.*, (2007) who reported that seropositive cases mainly showed high viral load in nasopharyngeal aspirate. As regards to sensitivity, specificity, positive predictive value and negative predictive value of ELISA were 84.2%, 100%,100% and 97.2%,our results are near to values recorded previously by Lindner *et al.*, (2008) and Zaghloul (2011).

Table.1 Demographic characteristics of patients with ALRT

Patients characteristics	(n=123)
*Sex: Male Female	 97 (78.86%) 26 (21.14%)
#Age, (months)	37 (7-59)
Duration of symptoms before admission (days)	7 (1–13)
Duration of hospitalization stay (days)	4.5 (1-9)

Data are represented as median (range),mean# or No (%)*

Table.2 Diagnostic efficiency HBo V IgM antibodies assay for detection of bocavirus

	Real- time PCR		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
HBoV IgM assay	Positive	Negative				
Positive	16	0	84.2%	100%	100%	97.2%
Negative	3	104				

PPV= positive predictive value, NPV= negative predictive value

Table.3 Clinical characteristics and serology of children infected by human bocavirus (HBoV) with high virus load and low virus load

Parameters	High Viral Load (n=13)	Low Viral Load (n= 6)	P value
Fever	9 (69.2%)	2 (33.33%)	0.319
Cough	9 (69.2%)	1 (16.7%)	0.058
Wheezing	12 (92.3%)	2 (33.33%)	0.017*
Tachycardia	10(76.9%)	1(16.7%)	0.041
Tachypnea	9 (69.2%)	1 (16.7%)	0.058
Dyspnea	2 (15.4%)	4 (66.7%)	0.046*
Rhinorrhea	9 (69.2%)	2 (33.33%)	0.319
Vomiting/diarrhea	4 (30.7%)	1 (16.7%)	0.631
#Duration of hospitalization stay (days)	5 (4-9)	3 (2-5)	0.002*
HBo V IgM antibodies +ve	13 (100%)	3 (50%)	0.02*

(Data are represented as No (%) or median (range) #, * Significant value)

Fig.1 The Age distribution of patients with bocavirus infection

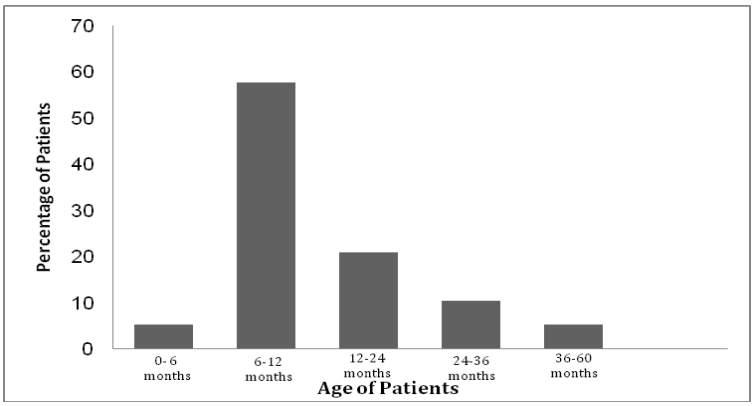
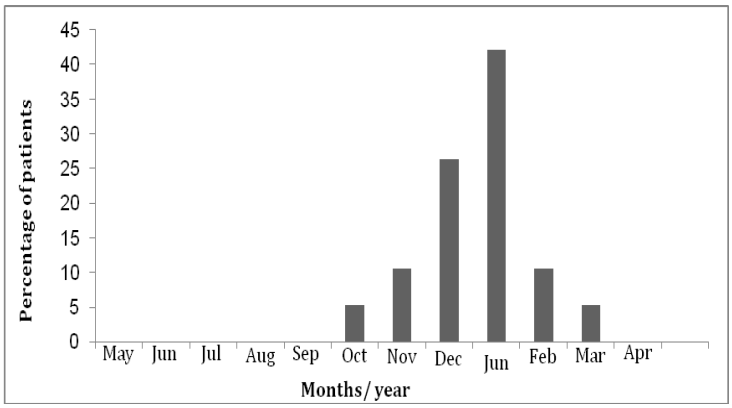


Fig.2 Seasonal distribution of human bocavirus infection during the study period among 2015 -2016



In conclusion, human bocavirus is an important cause of ALRI in young children less than five years; its incidence is highest in winter months. We used PCR and ELISA for the diagnosis of this virus, there was a high significant association between both methods that can confirm its role in ALRI and suggest active HBoV infection. There was an association between disease severity and HBoV viral load that is considered to be another clue for the role of HBoV as a causative agent of ALRI in young children less than five years. Wheezing and dyspnea were the most common clinical symptoms accompanied with high viral load; there presence may alert the pediatrician to investigate the presence of HBoV as a cause of ALRI. The high sensitivity and specificity of HBoV IgM detection by ELISA in comparison to real-time PCR is encouraging to recommend the usage of this easy non-sophisticated technique for diagnosis of HBoV infection in a wider scale.

An important limitation of this study was the relatively small number ALRI cases encountered during the time of the study, further larger study with the investigation of the association of HBoV with other respiratory viruses causing ALRI is recommended. To the best of our knowledge, this is the first study of the role of HBoV infection in ALRTI in children conducted in El Sharkia governorate, Egypt.

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