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# A novel primer set for improved direct gene sequencing of human bocavirus genotype-1 from clinical samples

Ahmed S. Abdel-Moneim[a,b,*], Mahmoud M. Kamel[c], Dina H. Hamed[d], Safaa S. Hassan[c], May S. Soliman[e], Saleh A. Al-Quraishy[f], Amani A. El Kholy[e]

[a] Microbiology Department, College of Medicine, Taif University, Al-Taif 21944, Saudi Arabia
[b] Virology Department, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62511, Egypt
[c] Clinical Pathology Department, National Cancer Institute, Cairo University, Giza, Egypt
[d] Department of Paediatrics, Faculty of Medicine, Cairo University, Giza, Egypt
[e] Clinical Pathology Department, Faculty of Medicine, Cairo University, Giza, Egypt
[f] Zoology Department, College of Science, King Saud University, Saudi Arabia, P.O. Box 2455, Riyadh 11451, Saudi Arabia

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A B S T R A C T

Human bocavirus genotype (HBoV-1) is a parvovirus associated with respiratory tract infections in children with different degrees of severity. The current study intended to improve the direct gene sequencing of the HBoV-1 using a newly developed primer set. Screening the presence of human bocavirus infection among in-patients children suffering from lower respiratory tract infections was another aim of the current study. Nasopharyngeal swab samples from in-patients children suffering from lower respiratory tract infections were examined. The real-time polymerase chain reaction was used for the initial screening as a highly sensitive method to detect the HBoV. Genotyping of real-time positive samples was attempted by direct sequencing of PCR amplicons using NP, VP1/2 and the newly developed VP/NC primers. HBoV-1 was present in 56.8% of the examined children. The newly developed primer set successfully amplified all real-time PCR positive samples, however, the other primer pairs did not reliably detect real-time PCR positive samples. The gene sequences of the detected HBoV-1 showed conserved sequences to each other with a low rate of discrepancies. The high rate of infection and the similarity between the detected strains strongly suggest nosocomial infections.



## 1. Introduction

In 2005, human bocavirus (HBoV) emerged as a new human parvovirus member in the genus *Bocaparvovirus*, subfamily *Parvovirinae*, family *Parvoviridae*. Four different genotypes of HBoV (HBoV1–4) have been recorded to date (Allander et al., 2005; Arthur et al., 2009; Kapoor et al., 2010, 2009). HBoV-1 has been frequently detected in the upper and lower airways of young children and, less frequently, from the gastrointestinal tract (Arnold, 2010; Schildgen et al., 2008). The other bocaviruses (HBoV-2, 3, and 4) were detected in human stool samples from patients suffered from acute gastroenteritis (Arthur et al., 2009; Kapoor et al., 2010, 2009). HBoV-1 is linked mostly with respiratory diseases and viral DNA has been found in between 1.5% and 22.5% of children with acute respiratory infections (Abdel-Moneim et al., 2013; Allander, 2008; Martin et al., 2010). HBoV has been detected in many countries worldwide, as reviewed in (Lusebrink et al., 2009), including in different parts of the Middle East (Abdel-Moneim et al., 2013; Hindiyeh et al., 2008; Kaplan et al., 2006; Kapoor et al., 2010; Zaghloul, 2011).

Bocaviruses are single-stranded DNA viruses with an average genome size of 5309 nucleotides. Three open reading frames (ORF) have been found in the HBoV genome: (i) a non-structural protein (NS1), (ii) two capsid proteins (VP1 and VP2) and (iii) a non-structural protein (NP1) (Lusebrink et al., 2009). Amino acid variations are associated with the genes of the VP1/VP2 capsid proteins, while NS1 and NP1 were found to be conserved regions of the HBoV genome (Chieochansin et al., 2008). HBoV detection has mostly been performed on nasopharyngeal aspirates and swabs and relies largely on conventional (Allander et al., 2005; Arden et al., 2006; Bastien et al., 2006; Kesebir et al., 2006; Manning et al., 2006; Schildgen et al., 2008; Sloots et al., 2006) and real-time PCR (Manning et al., 2006; Tozer et al., 2009). Genotyping based on NS1, NP1 or VP1/2 (Allander et al., 2005; Kesebir et al., 2006; Sloots et al., 2006) are commonly used for detection and genotyping of HBoV. Differences in the assay sensitivity extremely affect the ability of a

* Corresponding author at: College of Medicine, Taif University, Al-Taif, Saudi Arabia. Tel.: +96 6599107854.
*E-mail addresses:* asa@tu.edu.sa, asa@bsu.edu.eg (A.S. Abdel-Moneim).

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0166-0934/

**Table 1**
Oligonucleotides used for the amplification of different genes of HBoV.

| Name | | Primer sequence | Amplicon length (bp) | Reference |
|---|---|---|---|---|
| NP1 | For | 5′-GAGCTCTGTAAGTACTATTAC-3′ | 354 | Allander et al. (2005) |
| | Rev | 5′-CTCTGTGTTGACTGAATACAG-3′ | | |
| VP1-2 | For | 5′-GGACCACAGTCATCAGAC-3′ | 820 | Kesebir et al. (2006) |
| | Rev | 5′-CCACTACCATCGGGCTG-3′ | | |
| VP/NC | For | 5′-AGCTGTGAGATTGTATGGGAAG-3′ | 432 | This study |
| | Rev | 5′-TGTACAACAACAACACATTAAAAGATA-3′ | | |

certain assay to efficiently detect the amplicons in clinical samples (Allander et al., 2007).

The current study was intended to screen the presence of HBoV in in-patient children suffering from lower respiratory tract infection by using both commercial real-time PCR and conventional PCR using NP1, VP1/2 and the newly developed VP/NC primer sets and sequencing of PCR amplicons from HBoV positive patients.

## 2. Materials and methods

### 2.1. Ethics statement

The ethics committee of the Abo-Alreesh Paediatric Hospital approved the study protocol and the parents of the subject participants provided written informed consent.

### 2.2. Population study

The current study was conducted on 95 in-patient children from Abo-Alreesh Paediatric Hospital, Cairo, Egypt between December 2013 and February 2014. The children were all less than 36 months of age and showed manifestations of lower respiratory tract infection of varying degrees of severity. Individual swab samples were kept in a 1 ml universal virus transport medium (UTM$^{TM}$, COPAN, USA). The swab tip was cut off in the UTM and the tubes were transported to the lab in an ice box. Swabs were routinely processed and kept at −80 °C until further analysis.

### 2.3. Real-time polymerase chain reaction

A viral DNA/RNA extraction kit (Koma Bioteck Inc., Seoul, Korea) was used to extract viral nucleic acid from 100 μl of individual nasopharyngeal swab samples, according to the manufacturer's instructions. Extracted nucleic acid from individual samples were subjected to real-time PCR with commercial HBoV real-time kit (Life River, Shanghai, China) according to the manufacturer's instructions in Eppendorf Mastercycler® ep realplex2 and approved white PCR plastic ware (Eppendorf, Germany). The baseline fluorescence was automatically determined by the realplex software (Eppendorf, Germany). The cut-off for the quantification cycle threshold ($C_t$) determination was automatically calculated as 20 times the standard deviation of the fluorescence value of the baseline in cycles 5 through to 9. Each fluorescent reporter signal from the FAM channel was measured against the internal reference dye (JOE) signal in order to normalize the non-PCR-related fluorescence fluctuations between samples. All runs included the negative and positive controls which had been provided in the kits.

### 2.4. Genotyping of real-time PCR positive samples

Real-time PCR positive samples were subjected to conventional PCR amplification using the NP1, VP1/VP2 and the newly developed VP/NC primers. The new developed primer, flanks the 3′ end of the VP1/VP2 gene and the non-coding region (NC) of the HBoV genome, was designed using Primer3Plus (Rozen and Skaletsky, 2000) based on the HBoV KU3 strain (JQ411251). The free energies ($\Delta G$) associated with the possible secondary structures of each primer were analysed using OligoAnalyzer 3.1 (http://eu.idtdna.com/calc/analyzer). The primer set that showed positive $\Delta G$ was selected as the candidate primer set in the current study. The PCR amplification was conducted using a PCR master mix (Solis BioDyne, Inc., Estonia) and 20 pmol of each primer pair (Table 1). The thermal cycling conditions were as follows: an initial denaturation of 5 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 54 °C and 2 min at 72 °C, and a final extension of 10 min at 72 °C. For the newly developed VP/NC, 50 °C annealing temperature was used. In each run, both negative and positive controls were included. Positive PCR amplicons were purified using a gel extraction kit (Koma Bioteck, Inc., Seoul, Korea) and sequenced commercially (Macrogen Inc., Seoul, Korea).

### 2.5. Sequence analysis

The nucleotide sequences of NP1, VP1/VP2 and VP/NC sequences were compared with the equivalent HBoV sequences available from GenBank. Phylogenetic analyses were conducted using MEGA, version 5.1. Different HBoV gene sequences were submitted to GenBank (accession numbers KM577333–KM577342).

## 3. Results

### 3.1. Population study

A total of 95 nasopharyngeal samples were taken from in-patient children suffering from varying degrees of lower respiratory tract infection. Twenty-seven out of the 95 patients were in the intensive care unit (ICU) while the rest were in the regular in-patient section of the hospital (Table 2).

### 3.2. Real-time PCR

Fifty-four out of ninety-five children were found to be positive for HBoV using real-time PCR. The fifty-four positive samples included: 11/27 of the ICU patients and 43/68 of the in-patients (Fig. 1). The eleven ICU HBoV positive patients were suffering from either respiratory failure ($n$: 8) or bronchopneumonia ($n$: 3), while other HBoV positive in-patients were suffering from either bronchopneumonia ($n$: 39), pneumonia ($n$: 1) or bronchiolitis ($n$: 3).

### 3.3. Genotyping of real-time PCR positive samples

All real-time PCR positive samples were confirmed by direct gene sequencing of different PCR amplicons. The commonly used primers, NP1 and VP1/2, were able only to detect 3/54 real-time PCR positive samples with a sensitivity limit of $\geq 10^6$ copies/ml. Meanwhile, the newly developed set of primers succeeded to detect all the real-time PCR positive samples that showed $\geq 10^{3.3}$ copies/ml thus confirming its enhanced sensitivity (Fig. 1).

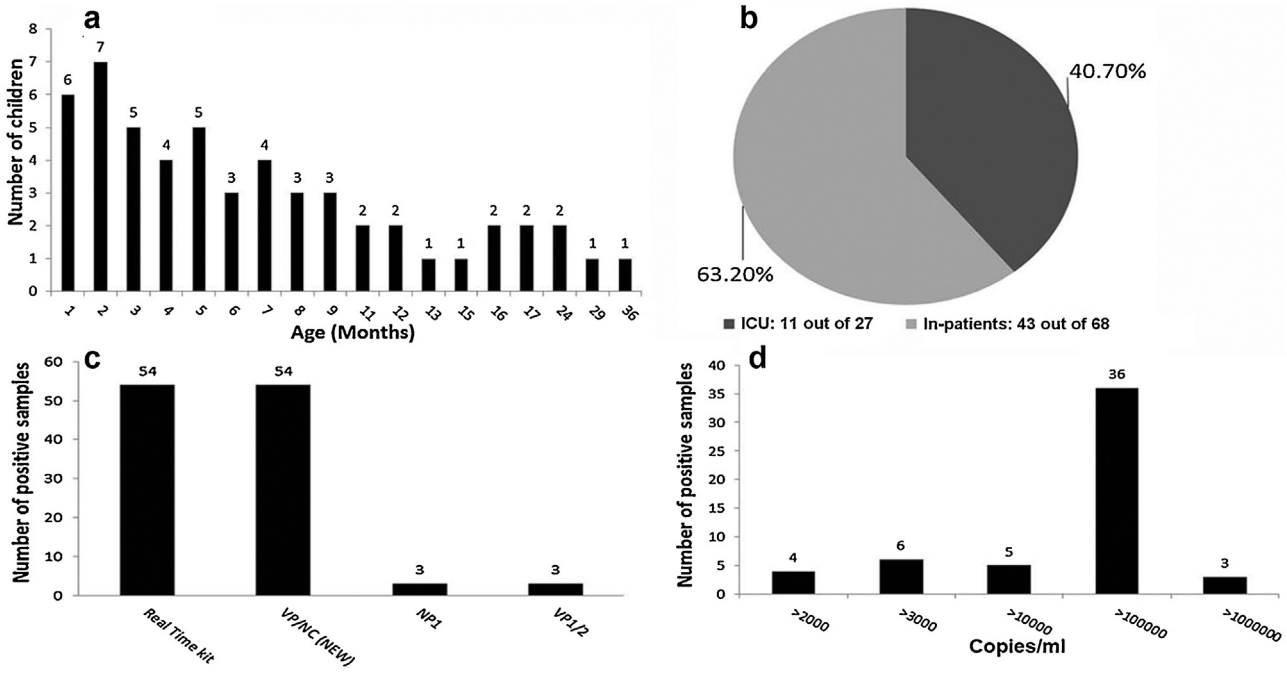


**Fig. 1.** Detection of bocavirus among children suffering from LRTI. (a) Bocavirus infections among different age groups. (b) HBoV infection in ICU and regular in-patient children. (c) The sensitivity of different assays for virus detection. (d) Number of copies/ml of different positive samples.

The phylogenetic trees of different gene sequences showed that the Egyptian strains are related to the HBoV-1 genotype (Fig. 2). Multi-sequence alignment of the Egyptian NP1 gene sequences were found to be identical to each other (data not shown). Nucleotide variations among the Egyptian strains were detected on the basis of VP1/VP2 and the newly developed VP/NC pair of primers. Interestingly, all the detected nucleotide variations among the Egyptian strains (Figs. 3 and 4) were silent mutations that did not result in any amino acid substitutions in the VP1/VP2 proteins.

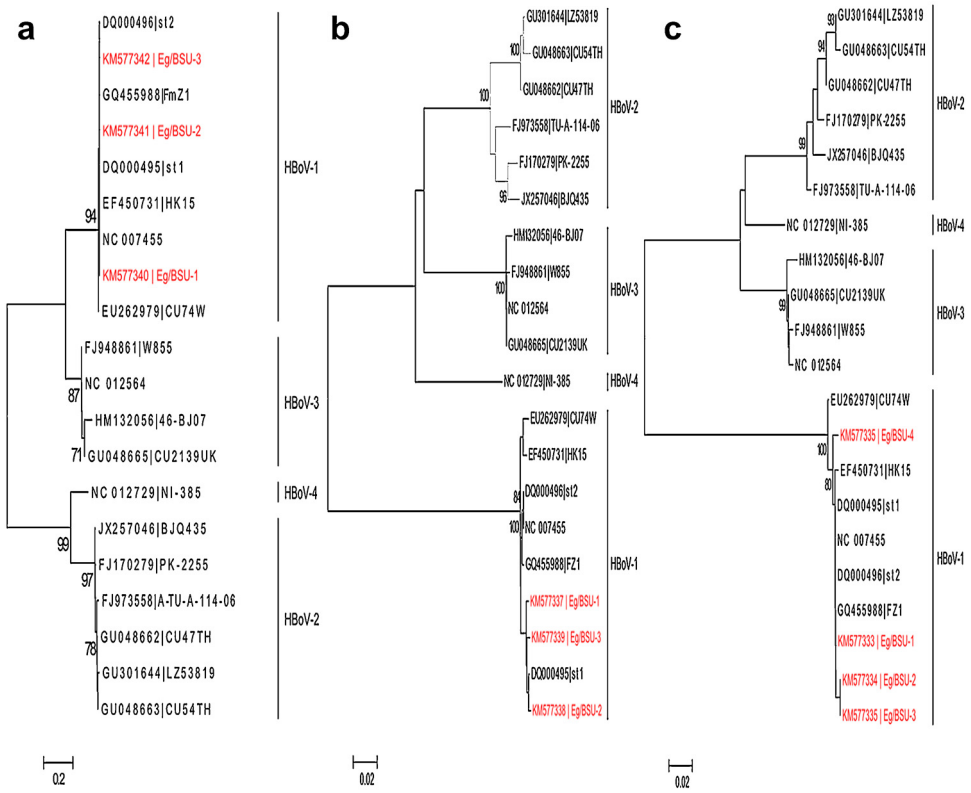


**Fig. 2.** Phylogenetic trees of HBoV. (a) NP1, (b) VP1/VP2 and (c) VP/NC gene nucleotide sequences in comparison to relevant sequences. The nearest-neighbour joining method (1000 bootstraps) was used to compare Egyptian HBoV nucleotide sequences (red colour) with other sequences from the GenBank databases.

```
                |1333 [a]
DQ000495|st1      ACTGCTCCAT TCATGGTTTG CACTAACCCA GAAGGAACAC ACATAAACAC AGGTGCTGCA GGATTTGGAT CTGGCTTTGA TCCTCCAAGC GGATGTCTGG
KM577337|Eg/BSU-1 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
KM577338|Eg/BSU-2 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
KM577339|Eg/BSU-3 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                |1433
DQ000495|st1      CACCAACTAA CCTAGAATAC AAACTTCAGT GGTACCAGAC ACCAGAAGGA ACAGGAAATA ATGGAAACAT AATTGCAAAC CCATCACTCT CAATGCTTAG
KM577337|Eg/BSU-1 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
KM577338|Eg/BSU-2 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
KM577339|Eg/BSU-3 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                |1533                       |1560
DQ000495|st1      AGACCAACTC CTATACAAAG GAAACCAGAC CACATACAAT CTAGTGGGGG ACATATGGAT GTTTCCAAAT CAAGTCTGGG ACAGATTTCC TATCACCAGA
KM577337|Eg/BSU-1 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
KM577338|Eg/BSU-2 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
KM577339|Eg/BSU-3 .......... .......... .......A.. .......... .......... .......... .......... .......... .......... ..........

                |1633                         |1662
DQ000495|st1      GAAAATCCAA TCTGGTGCAA AAAACCAAGG GCTGACAAAC ACACAATCAT GGATCCATTT GATGGATCCA TTGCAATGGA TCATCCTCCA GGCACTATTT
KM577337|Eg/BSU-1 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
KM577338|Eg/BSU-2 .......... .......... .........A .......... .......... .......... .......... .......... .......... ..........
KM577339|Eg/BSU-3 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                |1733                      |1758                          |1785                                  |1821
DQ000495|st1      TTATAAAAAT GGCAAAAATT CCAGTACCAA CTGCAACAAA TGCAGACTCA TATCTAAACA TATACTGTAC TGGACAAGTC AGCTGTGAAA TTGTATGGGA
KM577337|Eg/BSU-1 .......... .......... .....T.... .......... .......... .......... .......... .......... .......... ..........
KM577338|Eg/BSU-2 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
KM577339|Eg/BSU-3 .......... .......... .......... .......... .......... ..C....... .......... .......... ........G. ..........

                |1833
DQ000495|st1      AGTAGAAAGA TACGCAACAA AGAACTGGCG TCCAGAAAGA AGACATACTG CACTCGGGAT GTCACTGGGA GGAGAGAGCA ACTACACGCC TACATACCAC
KM577337|Eg/BSU-1 G......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
KM577338|Eg/BSU-2 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
KM577339|Eg/BSU-3 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

                |1933                                        |1977                                 End of VP1||Beginning of NCR [b]
DQ000495|st1      GTGGATCCAA CAGGAGCATA CATCCAGCCC ACGTCATATG ATCAGTGTAT GCCAGTAAAA ACAAACATCA ATAAAGTGTT GTAATCTTAT AAGCCTCTTT
KM577337|Eg/BSU-1 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
KM577338|Eg/BSU-2 .......... .......... .......... .......... ....A..... .......... .......... .......... .......... ..........
KM577339|Eg/BSU-3 .......... .......... .......... .......... ....A..... .......... .......... .......... .......... ..........

                |17                                                                                                  |101
DQ000495|st1      TTTGCTTCTG CTTACAAGTT CCTCCTCAAT GGACAAGCGG AAAGTGAAGG GTGACTGTAG TCCTGAGCTC ATGGGTTCAA GACCA
KM577337|Eg/BSU-1 .......... .......... .......... .......... .......... .......... .......... .......... .....
KM577338|Eg/BSU-2 .......... .......... .......... .......... .......... .......... .......... .......... .....
KM577339|Eg/BSU-3 .......... .......... .......... .......... .......... .......... .......... .......... .....
```

**Fig. 3.** Deduced nucleotide sequences of the VP1/VP2 gene of different Egyptian strains in comparison to the standard Swedish strain. (a) Nucleotide numbering is based on the VP1 sequence of st1 human bocavirus strain (Acc. No. DQ000495). (b) NCR: Non-coding region.

```
                |1835[a]                                |1869
DQ000495|st1      TAGAAAGATA CGCAACAAAG AACTGGCGTC CAGAAAGAAG ACATACTGCA CTCGGGATGT CACTGGGAGG AGAGAGCAAC TACACGCCTA CATACCACGT
KM577333|Eg/BSU-1 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
KM577334|Eg/BSU-2 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
KM577335|Eg/BSU-3 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
KM577336|Eg/BSU-4 .......... .......... .......... ....G..... .......... .......... .......... .......... .......... ..........
                |1935                                      |1977                                 End of VP1||Beginning of NCR[b]
DQ000495|st1      GGATCCAACA GGAGCATACA TCCAGCCCAC GTCATATGAT CAGTGTATGC CAGTAAAAAC AAACATCAAT AAAGTGTTGT AATCTTATAA GCCTCTTTTT
KM577333|Eg/BSU-1 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
KM577334|Eg/BSU-2 .......... .......... .......... .......... ..A....... .......... .......... .......... .......... ..........
KM577335|Eg/BSU-3 .......... .......... .......... .......... ..A....... .......... .......... .......... .......... ..........
KM577336|Eg/BSU-4 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
                |19                                                          |76
DQ000495|st1      TGCTTCTGCT TACAAGTTCC TCCTCAATGG ACAAGCGGAA AGTGAAGGGT GACTGTAGTC CTGAGCTCAT GGGTTCAAGA CCACAGCCCG ATGGTAGTGG
KM577333|Eg/BSU-1 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
KM577334|Eg/BSU-2 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
KM577335|Eg/BSU-3 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
KM577336|Eg/BSU-4 .......... .......... .......... .......... .......... .......C.. .......... .......... .......... ..........
                |119                                                                                                 |201
DQ000495|st1      TGTTACCGTC TCGAACCTAG CCGACAGCCC TTGTACATTG TGGGGGGAGC TGTTTTGTTT GCTTATGCAA TCGCGAAACT CTA
KM577333|Eg/BSU-1 .......... .......... .......... .......... .......... .......... .......... .......... ...
KM577334|Eg/BSU-2 .......... .......... .......... .......... .......... .......... .......... .......... ...
KM577335|Eg/BSU-3 .......... .......... .......... .......... .......... .......... .......... .......... ...
KM577336|Eg/BSU-4 .......... .......... .......... .......... .......... .......... .......... .......... ...
```

**Fig. 4.** Deduced nucleotide sequences of the VP/NC gene of different Egyptian strains in comparison to the standard Swedish strain. (a) Nucleotide numbering is based on the VP1 sequence of st1 human bocavirus strain (Acc. No. DQ000495). (b) NCR: Non-coding region.

## 4. Discussion

Primary infection with HBoV commonly occurs early in the life between the ages of 6–24 months (Abdel-Moneim et al., 2013; Ma et al., 2006; Volz et al., 2007), however, infections of older children and adults have also been recorded (Garbino et al., 2009; Longtin et al., 2008). New-borns are protected by maternally derived anti-HBoV antibodies that reduce virus infectivity (Ma et al., 2006; Meriluoto et al., 2012), although in this study, HBoV was nonetheless detected in very young children: 1 month (*n*: 6), 2 months (*n*: 7), 3 months (*n*: 5), and 4 months of age (*n*: 4).

It has been postulated that HBoV has a role as an inducer of respiratory diseases either as the sole respiratory pathogen or as a concomitant infection (Fry et al., 2007; Kaida et al., 2014; Maggi

**Table 2**
Clinical and demographic characteristics as well as laboratory findings of children suffering from respiratory distress.

| | Clinical data | Number/ratio |
|---|---|---|
| Age | (Months) | 8.1 ± 0.85 |
| Sex | Male | 56 |
| | Female | 39 |
| Predisposing | | |
| Preexisting disease | Asthma | 4 |
| | Cardiac | 13 |
| | Nervous | 5 |
| | Asthma/cardiac | 4 |
| | Others | 4 |
| | None | 65 |
| Parent smoking | Yes | 21 |
| | No | 74 |
| Signs | | |
| Fever | Yes | 95 |
| | No | 0 |
| Cough | Yes | 95 |
| | No | 0 |
| Wheezing | Yes | 74 |
| | No | 21 |
| Sputum | Yes | 31 |
| | No | 64 |
| Hospitalization | Inpatients | 68 |
| | ICU [a] | 27 |
| Diagnosis (mortality) | Bronchiolitis | 23 (1/23) |
| | Bronchopneumonia | 49 (2/49) |
| | Pneumonia | 8 (3/8) |
| | Respiratory failure | 15 (8/15) |
| Outcome | Death | 14 |
| | Discharge | 81 |

[a] ICU: Intensive care unit.

et al., 2007; Martinez-Roig et al., 2015). A virus load of more than $10^6$ copies/ml has been suggested to distinguish high and low HBoV viral loads in nasopharyngeal samples. High viral loads are suggestive of HBoV as a single entity in the absence of other viral agents, while low viral loads are usually associated with co-detection of other viruses (Christensen et al., 2013, 2010; Jacques et al., 2008; Moesker et al., 2015; Zhou et al., 2014). In the current study, only three samples showed a high viral load, equal to or more than $10^6$ copies/ml, while the rest of the positive HBoV samples showed a low viral load.

The very high detection rate of HBoV-1 in the current study: 54/95 (56.8%), which is far beyond the previously reported rates of infection: 1.5 and 22.5% (Abdel-Moneim et al., 2013; Allander, 2008; Martin et al., 2010). Given this high infection rate and the fact that the examined subjects were all reported in in-patient children in the same hospital, strongly suggests that the infection is nosocomial. This assumption is confirmed by the finding that fifty out of the fifty-four strains were identical to each other (data not shown). Interestingly, HBoV has been found in a case of a possible nosocomial infection in a neonatal intensive care unit as a sole detected respiratory virus (Calvo et al., 2008).

It was clearly demonstrated that the newly developed primer set succeeded to genotype all real-time PCR positive samples in comparison to NP1 (Allander et al., 2005) and VP1/2 (Kesebir et al., 2006) which amplified only 3/54 real-time PCR positive samples. It was clearly demonstrated that the newly developed primer set used in the current study greatly enhanced the PCR assay sensitivity. Interestingly, the variability in the detection of children with respiratory tract infection could be related to the sensitivity of the testing PCR assays and viral loads in samples (Broccolo et al., 2015; Chen et al., 2014). Reduced assay sensitivity and low viral load may lead to false negative PCR result (Chen et al., 2014).

Although HBoV has been detected in Egypt (Zaghloul, 2011), there is no sequence data available from the Egyptian HBoV strains. In the current study, the Egyptian strains showed high sequence homology to the original isolate, ST1, identified by Allander et al. (2005) and to other HBoV-1 strains identified worldwide. The phylogenetic trees and multi-sequence alignment of different gene sequences showed that the Egyptian strains are related to the HBoV-1 genotype with low genetic variability. Meanwhile, silent mutations were the only detected nucleotide variations in the VP1/VP2. This finding confirmed earlier work that HBoV strains exhibited limited variations (Abdel-Moneim et al., 2013; Allander et al., 2005; Ma et al., 2006). This suggests that a single genetic lineage of HBoV is circulating in humans in Egypt.

In conclusion, direct gene sequencing using the newly developed primer set is a useful diagnostic platform to detect HBoV-1 with high sensitivity. The current study suggests that HBoV-1 constitutes a significant potential nosocomial infectious pathogen in young children. To our knowledge, this is the first report of HBoV sequence analysis in Egypt in children with LRTI.

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