The use of real time pcr approach for detecting human boca virus (HBoV)

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ABSTRACT

Human boca virus (HBoV) is a newly discovered parvovirus prevalent among children with acute wheezing and can cause systemic infection. Suggested model for boca virus infection in which high viral loads are potentially associated with respiratory symptoms and low viral loads indicate asymptomatic shedding. Therefore, quantitative polymerase chain reaction analysis could be used as a sensitive method for studying the human boca virus. Moreover, design specific primers for the detection of this virus support the potentiality to develop a multiplex real time- PCR assay. In this research, the whole genome of many HBoV virual strains was aligned, and specific oligonucleotide primers have been designed by the use of bioinformatics softwares. In addition, a real time-PCR protocol has been optimized for the detection of HBoV. Results generated from this investigation showed that all designed primers were capable of detecting low titration of the virus using real time PCR protocol. Moreover, comparison of the cycle threshold values generated from this protocol against other previously described protocols has been carried out.

Key words: *Human boca virus, real time PCR, detection, bioinformatics, whole genome.*

INTRODUCTION

Human bocavirus (HBoV) belongs to the genus *Bocavirus* that infect cattle and dogs–thus the name, derived from bovine and canin (Manning *et al*., 2006). It is a newly identified human parvovirus that was originally identified in the respiratory secretions of children with respiratory tract disease (Kesebir *et al*., 2006). HBoV is belonging to the family *Parvoviridae,* subfamily *Parvovirinae*, and genus *Bocavirus*, and was cloned by molecular screening of pooled human respiratory tract samples in 2005. HBoV has been detected in patients with respiratory tract infections in many countries by PCR or realtime PCR (Shirkoohi *et al*., 2010). Parvovirus has been found in children with respiratory tract illness in practically all areas of the world in which it has been investigated (Pérez-Trallero *et al*., 2007). This virus has not been replicated *in vitro*, and no animal model has been reported. This is a frequent problem with newly identified viruses, since the very reason for them being undetected until now is their resistance to detection by traditional, culture-based methods (Allander *et al*., 2005). It was the first virus identified by "molecular virus screening", a procedure based on DNase treatment of the

samples, random amplification and cloning, followed by large scale sequencing and bioinformatic analyses (Allander, 2008). Meanwhile, The availability of rapid and reliable molecular diagnostics would therefore aid future studies of this novel virus. To address this, two sensitive and specific real-time TaqMan PCR assays that target the HBoV NS1 and NP-1 genes were developed (Xiaoyan *et al.*, 2006).

The aim of the present study is to optimize a realtime PCR-based approach to detect HBoV in clinical and non-clinical samples.

MATERIALS AND METHODS

Primer design and analysis

In order to detect HBOV full genomes, specific oligonucleotide primers were designed. The HBoV full length has been obtained from the National Centre for Biotechnology information (NCBI) (www. ncbi.nlm.nih.gov). Primer design has been carried out by the use of primer 3 software (http://www. genome.wi.mit.edu/cgi-bin/primer/ primer3-www.cgi). Alignment of the HBoV strains genome has been carried out by the use of Bioedit software program (http://www.bioedit.com). Oligonucleotide primers used in this study are listed in Table (1). Moreover, Table (2) contains the accession number of the strains used in the bioinformatics analysis.

Real time-PCR analysis

Detection and quantification of HBoV positive control sample (kindly obtained from Dr. Tobias Allander, Dept. of Clinical Microbiology, Karolinska University Hospital, Stockholm, Sweden) has been carried out by the use of Platinum Taq (5U/µl) (Invitrogen, USA). The reaction consists of Buffer (10x), $MgCl_2$ (50mM), dNTP's (10mM), forward primer (20µM), reverse primer (20µM), SYBR Green dye 0.25 µl per reaction (Invitrogen, USA). Machine program (Miniopticon, Biorad, USA) was as follows: 5 min at 95 °C for activation of *Taq* DNA polymerase enzyme, followed by 40 cycles for amplification with a denaturing step at 95 °C for 15 sec., gradient annealing temperatures (from 55- 65 °C) were used in order to identifying the optimal degree. Fixed extension step of 1 min at 72 °C was used. Five DNA concentrations were used in this study; 17 Million copies, 1.7 Million copies, 0.0017 Million copies, 0.00017 Million copies, and 0.000017 Million copies. Melting curve analysis has been carried out between 90°C and 60°C in 15 second intervals.

Conventional PCR analysis

A conventional PCR was performed to ensure the obtained data. Gels were prepared according to the ordinary lab procedures and using the primers LNS1 and LNS2. Agarose gel electrophoresis was performed to separate the obtained bands. The resulted pattern was viewed on UV-transilluminator after being stained with ethidium bromide. The PCR conditions were as follows: 94°C for 9 min, followed by 35 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min.

RESULTS

Oligonucleotide primer analysis

Designed oligonucleotide primers have been tested against the HBOV genome by using Bioedit bioinformatics software. The results showed that the designed primers have been matched successfully to the HBoV genomes (Figure 1). Sensitivity tests were performed to assess the cycle threshold (Ct) against the four DNA concentrations used with primer pair LSN1 and LNS2. Table (3) and Table (4) represent the values of Ct against the gradient DNA concentrations used. Figure (2) and Fig. (3) demonstrate sensitivity test of

Table (1): Oligonucleotide primers designed and used in this study.

Primers name	Primers Sequence	AT	EPS (bp)
LNS 1st 200-620 F	5`CCACGCTTGTGGTGAGTCTA3`	58 °c	247
1st _200-620_ R	5`CCCAAAATGGCGATCTTCTA3`		
2nd _ 820-1320_ F	5`CAGTGGATCCTCTTCGCTTC3`	58 °c	213
2nd _820-1320_ R	5`GCCCTGGAATGACTTCGTTA3`		

AT: Annealing temperature, EPS: Estimated Product size

Table (2): Examples of HBOv strains used for the bioinformatics analysis of this study.

Strains name
gi|77125236|ref|NC_007455.1| Human bocavirus, complete genome
gi|161137737|gb|EU262979.1| Human bocavirus isolate CU74W, complete genome
gi|161137732|gb|EU262978.1| Human bocavirus isolate CU8N, complete genome
gi|149389721|gb|EF450740.1| Human bocavirus isolate HK24, complete genome
gi|149389716|gb|EF450739.1| Human bocavirus isolate HK23, complete genome
gi|149389711|gb|EF450738.1| Human bocavirus isolate HK22, complete genome
gi|149389706|gb|EF450737.1| Human bocavirus isolate HK21, complete genome
gi|149389701|gb|EF450736.1| Human bocavirus isolate HK20, complete genome
gi|149389696|gb|EF450735.1| Human bocavirus isolate HK19, complete genome
gi|149389691|gb|EF450734.1| Human bocavirus isolate HK18, complete genome
gi|149389686|gb|EF450733.1| Human bocavirus isolate HK17, complete genome
gi|149389681|gb|EF450732.1| Human bocavirus isolate HK16, complete genome
gi|149389676|gb|EF450731.1| Human bocavirus isolate HK15, complete genome
gi|149389671|gb|EF450730.1| Human bocavirus isolate HK14, complete genome
gi|149389666|gb|EF450729.1| Human bocavirus isolate HK13, complete genome
gi|149389661|gb|EF450728.1| Human bocavirus isolate HK12, complete genome
gi|149389656|gb|EF450727.1| Human bocavirus isolate HK11, complete genome
gi|149389651|gb|EF450726.1| Human bocavirus isolate HK10, complete genome
gi|149389646|gb|EF450725.1| Human bocavirus isolate HK9, complete genome

Table (3): Sensitivity test of the examined annealing temperature with primer LNS1.

Annealing Temperature °C	Tempelate conc.	C.t.
55	17 Million copy	14.51
58	17 Million copy	14.09
60	17 Million copy	14.13
63	17 Million copy	14.44
65	17 Million copy	14.77
55	1.7 Million copy	18.08
58	1.7 Million copy	16.91
60	1.7 Million copy	27.2
63	1.7 Million copy	N/A
65	1.7 Million copy	18.23
58	0.017 Million copy	23.74
60	0.017 Million copy	N/A
63	0.017 Million copy	25.07
55	0.0017 Million copy	28.92
58	0.0017 Million copy	28.46
60	0.0017 Million copy	27.81
63	0.0017 Million copy	27.29
65	0.0017 Million copy	28.20

Table (4): Sensitivity test of the examined annealing temperature with primer LNS2.

Annealing Temperature°C	Template Conc.	C.t.
55	17 Million copy	13.47
58	17 Million copy	13.39
60	17 Million copy	13.46
63	17 Million copy	13.65
65	17 Million copy	14.06
55	1.7 Million copy	17.87
58	1.7 Million copy	17.08
60	1.7 Million copy	17.28
63	1.7 Million copy	17.19
65	1.7 Million copy	17.51
55	0.17 Million copy	21.68
58	0.17 Million copy	21.08
60	0.17 Million copy	21.17
63	0.17 Million copy	21.25
65	0.17 Million copy	21.41
55	0.017 Million copy	24.50
58	0.017 Million copy	24.18
60	0.017 Million copy	24.37
63	0.017 Million copy	24.47
65	0.017 Million copy	25.14

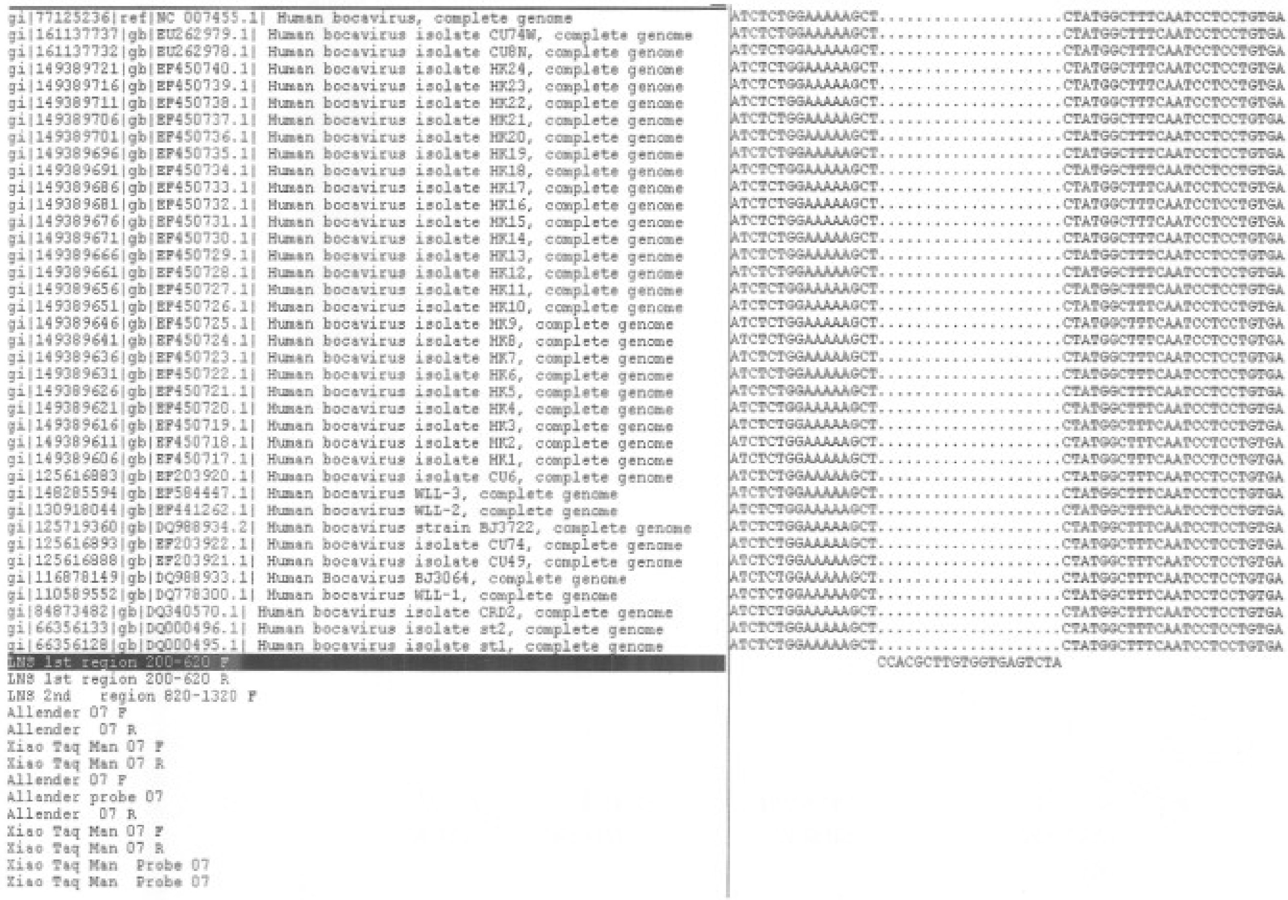


Fig. (1): Bioinformatics Analysis of primer as a Model of the bioinformatics analysis of the designed primers.

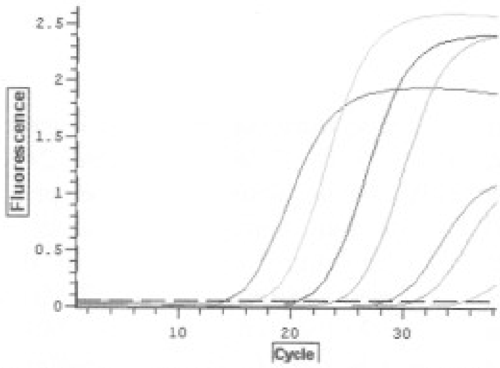


Fig. (2): Sensitivity test of primer LNS1 at the annealing temperature of 58 °C.

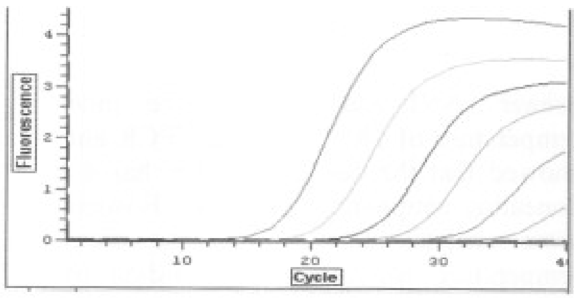


Fig. (3): Sensitivity test of primer LNS2 at the annealing temperature of 58 °C.

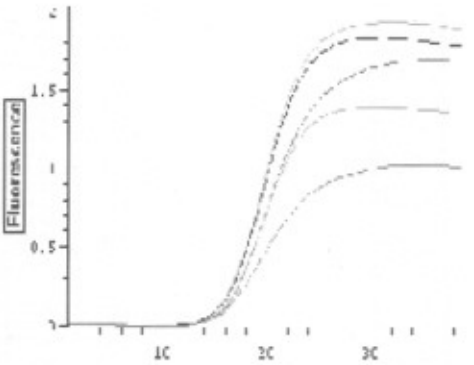


Fig. (4): Optimization of annealing temperature of LNS1 primer; Well A1: 55 °C, A5: 58 °C, A6: 60 °C, A7: 63 °C A10: 65 °C.

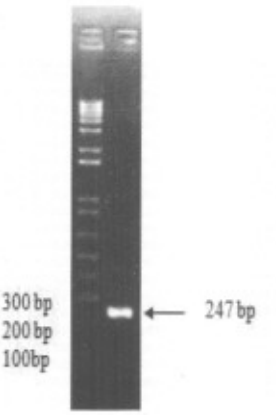
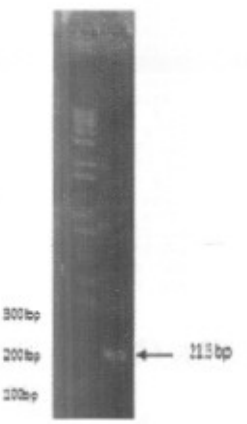


Fig. (5): PCR product generated by primer LNS1; Lane 1, Molecular weight standard; Lane 2, HBOv specific PCR product.

Fig. (6): PCR product generated by primer LNS2; Lane1, Molecular weight standard; Lane 2, HBOv specific PCR product.



primer LNS1 and LNS2 at the annealing temperature of 58 °C. In silico PCR analysis showed that the designed primer has a fixed annealing temperature of 58 °C. However, *in vitro* analysis showed that the annealing temperature was variable according to the DNA concentration (Figure 4 and Table 5). The data obtained indicated that in all the concentrations applied (0.017 Million copies 0.17 Million copies, 1.7 Million copies, and 17 Million copies) using the primer LNS2, the annealing temperature was 58 °C as it gave a Ct value of 13.39 17.08, 21.08, and 24.18, respectively. While when using the primer LNS1, in the four concentrations used, the annealing temperature was 58 °C in three concentrations (17 Million copies, 1.7 Million copies, 0.017 Million copies, 0.0017 Million copies) but it was 62°C in the concentration 0.0017 Million copies.

Real time PCR analysis

Real time PCR analysis for the detection of HBoV has been carried out. The results (indicated as cycle threshold (Ct) values) showed that the primer LNS1 was the best as it gave a Ct value of 13.94 followed by primer LNS3 which gave a Ct value of 14.03. Sensitivity analysis of this protocol showed that there is a gradient increase in the Ct values in response to the gradient decrease of the virus DNA concentration. Also, by the use of primer LNS1, the recommended protocol was capable of detecting a very low viral concentration down to (0.0000017 µg). However, the sensitivity limit of primer LNS2 was (0.000017 µg) (Table 6).

Agarose gel electrophoresis

To visualize the obtained results, ten microliters of each amplified product was loaded in an agarose gel (2.5%) containing 0.5 ug/ml ethidium bromide and then electrophoresed in TBE (89 mM Tris-borate 2 mM EDTA, pH 8.0) buffer. After the electrophoresis the DNA bands were visualized by UV-transilluminator. The data generated were photographed and then subjected to analyses by Gel documentation system (Gel Pro Analyzer version 3.1).

Conventional PCR analysis

Conventional PCR was performed to ensure the data gained by the real time PCR and the in slico PCR. The obtained data (Figure 5 and Figure 6) showed that using the primer sets for LNS1 and LNS2 amplified a fragment with the expected molecular weight.

DISCUSSION

To date, the detection of HBoV has been performed predominantly on NPAs and swabs and has been possible only with PCR-based methods (Schildgen *et al.*, 2008). Since the described oligonucleotide primers have been designed based on the sequence alignment analysis, the absence of any mismatches of these primers against the tested HBoV strains was expected. Moreover, this result also could be attributed to the relatively small number of HBoV strains that have been completely sequenced and published at NCBI. Designed primers have been tested against the whole length genome of HBOV genome and informative patterns have been obtained. Among five primers designed (data not shown), only two primers (LNS1 and LNS2) were the most powerful oligonucleotides in generating a specific profile against the genome of HBOV. Sensitivity tests were performed to assess the Ct values and to assess also the best annealing temperature (55-65° C) against the four viral DNA concentrations (17 Million copies, 1.7 Million copies, 0.17 Million copies, 0.017 Million copies, 0.017 Million copies, 0.0017 Million copies, and 0.00017 Million copies). The data indicated that the annealing temperature 58 °C was dominated over the other temperatures used against the viral DNA concentrations of 17 Million copies, 1.7 Million copies, and 0.017 Million copies, as this temperature gave a Ct values of 14.09, 16.91, and 23.74. This may be in disagreement with the findings obtained with the in silico PCR which determined the optimum annealing temperature of 55 °C against a wide range of viral DNA copy number. Meanwhile the Ct values of the designed primers LNS1 and LNS2 at the annealing temperatures 58 °C was 14.09 and 13.39, respectively, indicating that this annealing temperature was the most appropriate one among the range employed in this study. However the designed primers LNS1 and LNS2 might be used in PCR amplification likewise in sequencing as the primer length was almost the same as that used by Xiaoming *et al.* (2006) who used the primer F 5- GAGCTCTGTAAGTACTATTAC-3 and R

5- TCTGTGTTGACTGAATAC-3 for both amplification and sequencing.

Conventional PCR analysis

Conventional PCR was performed according to the ordinary lab procedures and band with molecular weight of 247 and 213 bp were obtained when LNS1 and LNS2 primers were used, respectively. According to the primers used to generate the specific profile different molecular weight bands could be observed. Kleines *et al.* (2007) identified a target band of 354 bp. The initial studies on hBoV infections have used conventional PCR methods with agarose gel electrophoresis (Neske *et al.*, 2007). The aim of our study was to establish a real-time PCR assay for the rapid detection and quantification of hBoV DNA. We developed a real time-based approach to detect the hBoV as a rapid and reliable method in comparison to the conventional PCR, however the real-time hBoV PCR was in good agreement with a conventional hBoV PCR. But because the amplified PCR fragment in the real-time PCR is shorter than in the conventional PCR, the real-time PCR may appear more sensitive. This assay proved to be sensitive, specific, and reliable for hBoV DNA amplification and quantification and was possible over a broad linear range. Results of real time PCR analysis showed that all designed oligonucleotide primers were efficient against the tested HBoV positive strain. However, optimization of real time PCR conditions was carried out. Several real time PCR conditions were tested. Although in silico PCR analysis recommended the use of 55 °C as an annealing temperature, *in vitro* experiments showed that the best annealing temperature is 58 °C. Also, sensitivity analysis of this protocol showed that there is a gradient increase in the Ct values in response to the gradient decrease of the virus concentration. These results indicated the reliability of the protocols employed in this study. Also, by the use of primer LNS, the recommended protocol was capable of detecting a very low viral concentration up to (0.0000017 µg). However, the sensitivity limit of primer LNS2 was (0.000017 µg). In conclusion, while in silico PCR analysis revealed that the best annealing temperature of primers designed in this study was 55 °C, *in vitro* experiments showed that the best annealing temperature was 58 °C. Also, sensitivity test of oligonucleotide primers used in this study showed that primer LNS1 is better than primer LNS2. However, these results suggest that a comparison of this protocol to the other recommended protocols should be carried out. Also, testing of this protocol against clinical samples will be of great value.

Table (5): Ct values of the designed primers at the selected annealing temperatures.

Primer	55 °C	58 °C	60 °C	63 °C	65 °C
LNS1	14.51	14.09	14.13	14.44	14.77
LNS2	13.47	13.39	13.46	13.65	14.06

Table (6): Cycle threshold (Ct) values of the oligonucleotide primers generated in this study.

Primer	Sample quantity (µg)	Ct value
LNS1	1.7	14.0
LNS2	1.7	14.0
LNS1	0.17	16.3
LNS2	0.17	19.4
LNS1	0.017	20.5
LNS2	0.017	23.1
LNS1	0.0017	23.7
LNS2	0.0017	33.9
LNS1	0.00017	28.2
LNS2	0.00017	30.3
LNS1	0.000017	30.6
LNS2	0.000017	33.5
LNS1	0.0000017	35.4
LNS2	0.0000017	N/A

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الملخص العربي

إستخدام تفاعل البلمرة المتسلسل التواقعي للكشف عن فيروس البوكا

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يعد فيروس البوكا الذى يصيب الإنسان من الفيروسات المكتشفة حديثا وهو فيروس يصيب الأطفال خصوصا مسببا عدوى جهازية. والنموذج المقترح حديثا للعدوى الناشئة عن هذا الفيروسات فى حالات الإصابة حال كون العد الفيروسى مرتفعا هى ارتباطه بظهور أعراض تنفسية أما فى حال كون العد الفيروسى منخفضا فتكون الآلية المقترحة هى عدم ظهور أعراض للمرض. وعلى ذلك فتحليلات تفاعل البلمرة المتسلسل الكمى تعد اتجاها هاما فى الكشف عن هذا الفيروس. كما أن تصميم بادئات متخصصة يسهم أيضا فى الكشف عن هذا الفيروس فى تفاعلات البلمرة المتسلسلة متعددة البادئات. وفى هذه الدراسة تم ترصيص جينومات العديد من سلالات فيروس البوكا لاستنتاج بادئات مناسبة وكان ذلك باستخدام أحد برامج المعلوماتية الحيوية. وقد أمكن تهنية نظام لتفاعل البلمرة المتسلسل يمكنه الكشف وبدقة عن هذا الفيروس. هذا وقد أوضحت النتائج المتحصل عليها من هذه الدراسة أن البادئات التى تم تصميمها أمكنها الكشف عن الحد الأدنى من جزيئات الفيروس. وفضلا عن ذلك فقد تمت مقارنة الحد الفاصل الذى يمكن عنده الكشف عن أقل عدد ممكن من جزيئات الفيروس مع ما تم فى دراسات سابقة.