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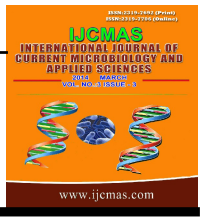



**Original Research Article**

# Quantification of *H. influenzae* Type b in cerebrospinal fluid from children with meningitis

**Majeed Arsheed Sabbah[1], Ghusoon A. Abdulhasan[2*], Kifah A. Jasem[3], and Hayfa H. Hassani[2]**

[1]Alnahrain University, Biotechnology research center, Iraq
[2]Department of Biology, College of science, University of Baghdad, Baghdad-Al-Jadiria, Iraq
[3]Ministry of health, Central health laboratory, Iraq
***Corresponding author***

**Keywords**

*Haemophilus influenzae;* CSF; real time PCR; meningitis; Iraq.

**A B S T R A C T**

*Haemophilus influenzae* type b capsule are associated with invasive diseases including meningitis. Development of specific and sensitive assay for detection and quantification of this pathogen have great impact in diagnosis and treatment. In this work, TaqMan real time PCR were developed for rapid identification and quantification of *H. influenzae* type b in cerebrospinal fluid specimens (CSF) collected from children less than 5 years old diagnosed with meningitis. Among 75 isolates and CSF specimens, 25(33.3%) were positive to *H. influenzae* type b using real time PCR. The copy number of *bcs3* in CSF was arranged from $88\times10^5$ to $7.5\times10^3$ copy/µl. Assessment of methods used for detection of *H. influenzae* in CSF was carried out. It was noticed that real time PCR had high sensitivity (100%) than traditional method (44%).

## Introduction

*Haemophilus influenzae*, a gram negative coccobacillus whose environmental niche is primarily restricted to the human respiratory tract, is classified on the basis of production of a polysaccharide capsule: strain types a through f produce antigenically distinct capsules that associated with invasive disease, and nontypeable strains produce no capsule (Gilsdorf *et al*., 2004). *H. influenzae* type b is responsible for most cases of invasive *H. influenzae* disease, including meningitis and septicemia, in unvaccinated children (Ito *et al*., 2011). The polysaccharide capsule of *H. influenzae* strains is encoded by ten genes located in a single locus called a cap locus. Cap locus consists of three distinct regions, all six capsular type have shared region 1 and 3 while region 2 involved in type specific capsule (Luong *et al*. 2004; Davis *et al*, 2011).

The molecular assay of *H. influenzae* was published in 1980s by using probing method for detecting *H. influenzae* (Malouin *et al*., 1988) and since then

several methods based on amplification of different target genes within capsular genes have been described. Capsular genes were suitable target for the detection and typing of capsulated *H. influenzae* by using PCR assay that shown to be highly sensitive method compared to bacterial culture (Maaroufi *et al*., 2007; Nelson and Smith, 2010).

However, the conventional PCR technique is laborious when large numbers of samples need to be analyzed. Also, the little quantity of *H. influenzae* DNA makes it difficult to detect by gel based electrophoresis. Thus, real- time PCR used to solve these problems which several applications of real-time PCR were used for detection and quantification of cultured *H. influenzae* or that found in clinical specimens have been reported (Roine *et al*., 2009; Abdeldaim *et al*., 2010; Sacchi *et al*., 2011).

In our previous work it was found that *H. influenzae* type b is the dominant type of *H. influenzae* in CSF specimens collected from children less than five years diagnosed with meningitis in Iraq, so this work was aimed to use real time PCR technique for a rapid identification and quantification of *H. influenzae* type b in CSF specimens.

## Materials and Methods

### Bacterial isolates and CSF specimens collection

Eleven cultured isolates of *H. influenzae* type b from CSF of previous study were used that identified morphologically, biochemically, and serologically. Sixty four CSF specimens were collected from children less than five years old from different Iraqi hospitals with probable meningitis who having symptoms with turbid CSF, elevated protein > 100mg/dl, decreased glucose<40mg/dl or WBC> 100mg/dl with more than 80% neutrophil as well as suspected meningitis who having symptoms of meningitis. One milliliter of CSF was transported to the Central Health Laboratory with ice bag and stored at -20 C until used. These specimens were collected from January and December 2010.

### Extraction of Genomic DNA

An overnight culture of bacteria were used for extraction of DNA by wizard genomic DNA purification kit (promega, USA) while 100 µl of CSF specimens was used for extraction of DNA Mini Kit (Geneaid, Thailand) according to manufacture instructions.

### Quantitative Real-Time PCR

### Primers, Probe selection, and their specificity testing

Cap region II type b specific gene (*bcs3)* of *H. influenzae* was selected for designation primers and probe manually from database of *H. influenzae* (GeneBank accession no. X78559) and synthesized in Bioneer (Korea). The forward primer (5´-ATACGACAGTATTCCTGATTAC-3´) and the reverse primer (5´-ATCTTCGTCTTGCGTATTGAG -3´) were used to amplify 147 bp fragment. The amplicon was detected with a TaqMan oligonucleotide probe (Fam-5´-ATCTTCGTCTTGCGTATTGAG -3´-TAMARA).

Primers and probe were tested for specificity via BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST), as well as the specificity was tested in

comparison with other bacteria caused meningitis including *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Staphylococcus aureus*. Primers were tested by conventional PCR with *H. influenzae* type b bacteria to check the correct size of the amplicon on 1.5% agarose gel electrophoresis and detect the presence or absence of primer dimmer.

**Absolute Standard Curve**

The standard curve for quantification of *bcs3* in CSF specimens was prepared by recovering of DNA fragments from gel and purified them using Gel/PCR DNA fragments extraction kit (Geneaid/ Tailand) according to manufacture instructions then determined the concentration of purified DNA.

Series of ten-fold dilutions (3×10 - $3\times10^5$ ng/µl) were prepared and run in duplicate using the ABI prism 7500 real-time PCR. The standard curve was generated by plotting Ct of dilutions against the log of the DNA concentration (Pau Ni et al., 2006).

The *bcs3* Copy number of the tested DNA was calculated using the Applied Biosystems protocol (2003). The quantity of positive CSF samples were converted from ng/ml to copy/µl by calculating the genome mass (m) when the molecular weight of 1 bp of DNA was 660 g/mole and genomic size of *H. influenza*e is 1830140 bp as the following:

$$m = (genomic\ size\ of\ bacteria)\left(\frac{1\ mole}{6.023\times10^{23}\ molecules}\right)\left(\frac{660\ g}{mole}\right)$$

**PCR Amplification**

PCR mixture was set up in a total volume of 25µl included TaqMan universal master mix, 2 pico/µl of each primer, 1 pico/µl of probe, and 2µl of 100 ng/µl template DNA. The rest volume was completed with sterile D.W. Quantitative real-time PCR was carried out in ABI prism 7500 real-time PCR. The reaction was initiated by activation of Taq polymerase at 95˚C for 10min, followed by 40 cycles consisting of 15sec denaturation at 95˚C and 1min annealing at 60˚C. PCR products were detected by measuring flourecenses and analyzed with applied biosystem software. Negative control was also prepared in parallel with each run.

**Statistical Analysis**

Data were presented as percentage and/or mean ± standard error. The Statistical Analysis System- SAS (2004) was used to analyse the effect of difference factors in this study. T-test was used to analyse the data. P value $\leq$ 0.05 was considered statistically significant.

## Results and Discussion

A real time PCR was used for quantitative detection of *H. influenza*e type b in 11 isolates of *H. influenza*e type b and 64 CSF specimens from children with meningitis.

Newly primers and probe showed high specificity via BLAST searches and non fluorescent signal was detected with other bacteria tested in this study which refer no cross reactivity with primers and probe under estimation. Moreover, a single band with 147bp in size without primmer dimmer was observed on ethidium bromide stained gel (Figure 1).

The absolute quantity of *H. influenzae* type b in CSF specimens was determined by standard curve of real-time PCR. The average of Ct values of standard curve was 17.6, 20, 23.5, 27.6, and 31 as seen in Figure 2 and 3. Moreover, this curve had a slope -3.468 which yielded high efficiency (94%) with high correlation coefficient (0.99).

The suitability of *bcs3* was used for diagnosis *H. influenzae* type b in CSF specimens by real-time PCR. The sample was considered positive for *H. influenzae* type b DNA when the exponential shape increased in fluorescence during the first 35 cycles of amplification, whereas the negative result was assigned as no amplification when the Ct value was greater than 40. If the Ct value was between 35 and 40, the sample was diluted 10 fold due to present of inhibitors (Wang *et al*., 2011).

Among 75 isolates and CSF specimens, 25(33.3%) were positive to *H. influenzae* type b using this technique and the amplification curves had an exponential shapes. On the other hand, no amplification was observed in control group and negative control.

The copy number of *bcs3* in CSF specimens was determined by calculation the genome mass of *H. influenzae.* The mass of *H. influenzae* genome was 2 fg which represented 1 copy of gene. Thus 1 ng of genomic DNA contains $5\times10^5$ copies. The copy number of *bcs3* in probable and suspected groups were $88\times10^5$ copy/µl (log: 5.22±0.7) and $7.5\times10^3$ copy/µl (log: 3.65±0.21), respectively, which showed Ct value between 19 to 35 cycles.

Sensitivity and specificity of traditional methods for identification *H. influenzae* type b in comparison with real-time PCR technique were 44% and 100% respectively (Table 1).

In previous study we showed that *H. influenzae* type b was the most dominant type in CSF samples collected from children less than five years old diagnosed with meningitis. Marty *et al*. (2004) and Maaroufi *et al*. (2007) observed that all invasive *H. influenzae* isolates detected as *H. influenzae* type b by TaqMan real-time PCR and these results were in fill agreement with PCR capsular genotyping results.

In this study real time PCR was developed for identification and quantification of *H. influenzae* type b due to its high sensitivity and efficiency. As known, the efficacy of real-time PCR may return to the amplification of target DNA under short time condition (Dagher *et al*., 2004) as well as detection of products by real-time PCR occurs during the log phase instead of the plateau phase which gives more accurate quantification of target concentration (Pau Ni *et al*., 2006).

Determination of gene copy number is so important for absolute quantification. Roine et al. (2009) found that the mean of CSF genome of *H. influenza*e type b meningitis was $3.5\times10^3$ copy/µl when used *bcs3* as target gene in real-time PCR. However, the copy number of *bcs3* in this technique does not represent the number of bacteria found in CSF because most invasive *H. influenza*e type b strains possess a duplication of the capsulation locus and may have more than two copies as reported by Cerquetti *et al*. (2005). Therefore, there is a possibility that high *H. influenza*e type b genome count in this study may return to the presence of two or more copies of the *H. influenza*e type b

**Table.1** Comparison of traditional methods with real-time PCR technique for detection of *H. influenzae* type b from CSF of meningitis cases

| Test | | Real-Time PCR | | Total N=75 | Sensitivity * | Specificity ** |
|---|---|---|---|---|---|---|
| | | N=23 + | N=52 - | | | |
| Traditional methods | + | 11 | 0 | 11 | 44% | 100% |
| | - | 14 | 50 | 64 | | |

* **Sensitivity** was defined as the number of true positives/ (the number of true positives+ the number of false negatives).
** **Specificity** was defined as the number of true negatives/ (the number of true negatives + the number of false positives).

**Figure.1** Agarose gel (1.5%) electrophoresis (5 V/cm for 2 hrs) of amplified *bcs3* (147pb) from *H. influenza*e type b DNA stained with ethidium bromide. Lane 1. 100 bp DNA ladder, Lane 2-5. *H. influenza*e type b isolates, Lane 6. Negative control (had all PCR mixture including water instead of DNA template).

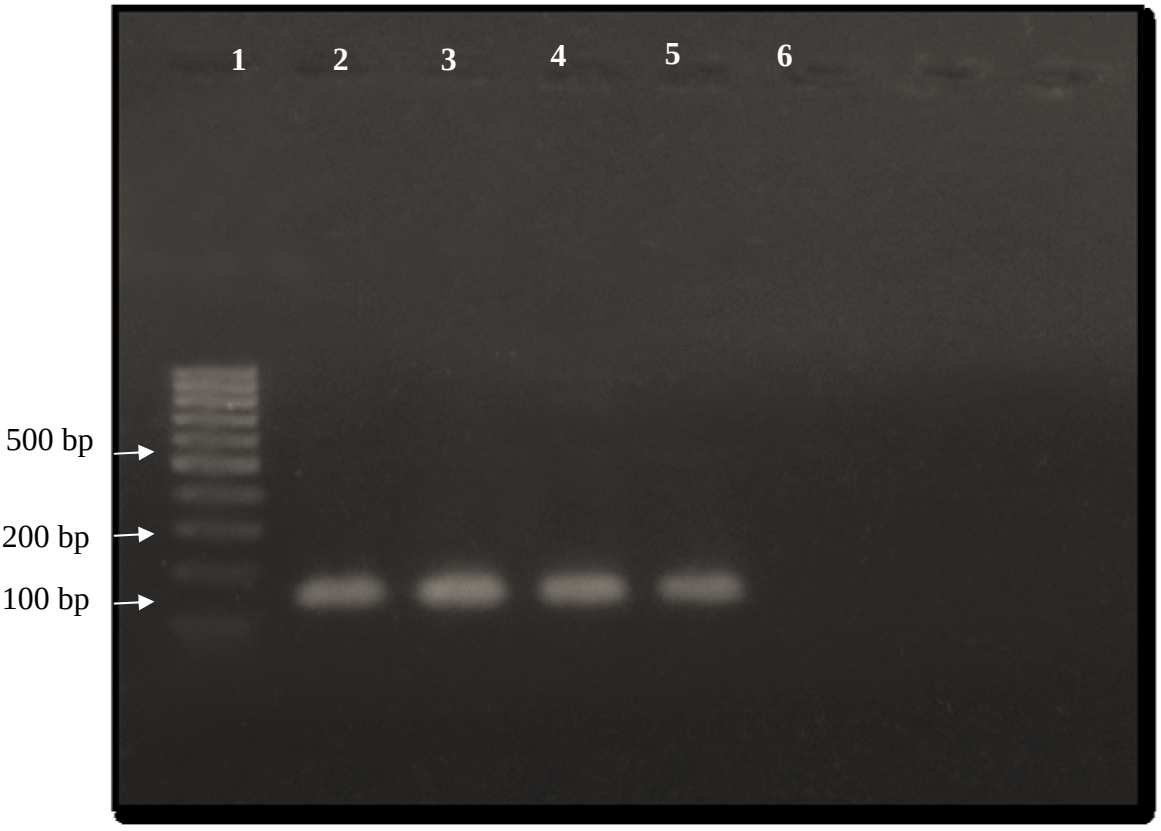


**Figure.2** Amplification plot of standard curve by real-time PCR for 5 serial concentrations ($3\times10$ - $3\times10^5$ ng/ml) of purified *bcs3*.

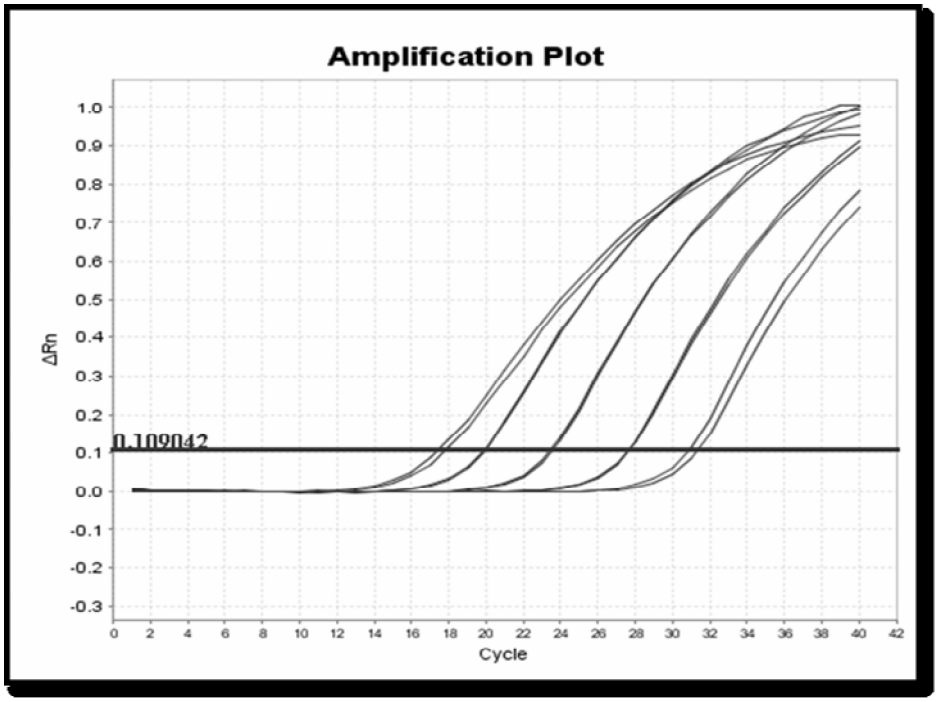
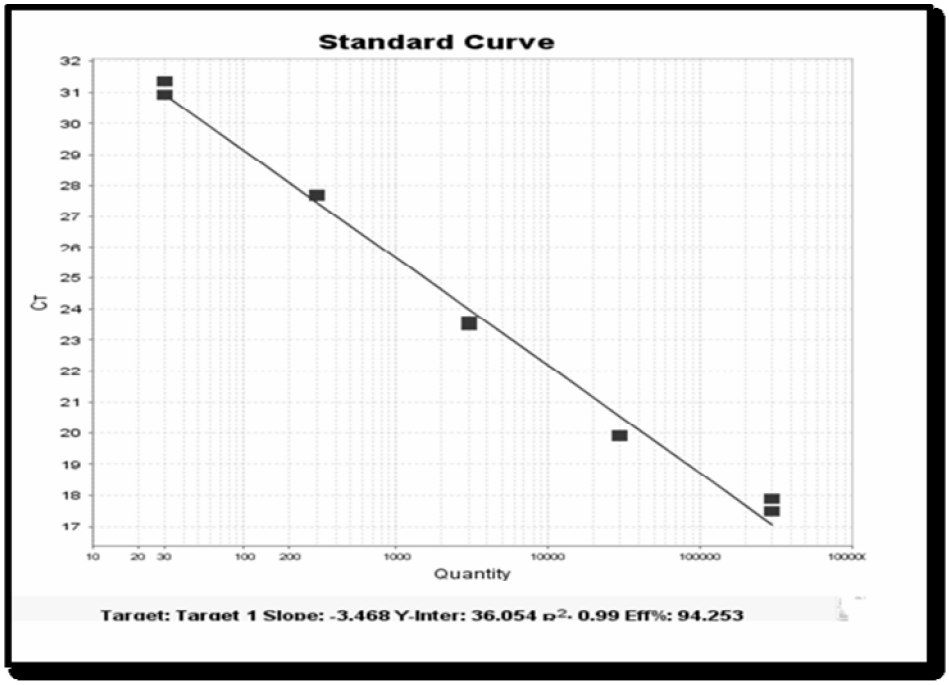



**Figure.3** Comparison of standard curve of Ct plotted against various concentrations of *bcs3* from *H. influenzae* type b. X-axis denotes the series concentration of purified *bcs3*and Y-axis represents the cycle number



capsular locus but this phenomenon increased the sensitivity of PCR by targeting genes with multiple copies as seen in detecting *H. influenza*e type b by using *bcs3* (Roine *et al*., 2009) and *hly* gene for detecting *Listeria monocytogenes* in CSF (Monnier *et al*., 2011).

The specificity of traditional methods was high (100%) but the sensitivity was low (44%) when compared with real-time PCR techniques for diagnosis *H. influenza*e type b in CSF specimens. This finding was supported by previously published data concerning the high sensitivity and specificity of PCR assay (Nakhjavani *et al*., 2005; *Tuyama et al*., 2008). In addition, several investigators pointed the poor sensitivity of culture in identification of bacterial meningitis, particularly in the presence of antibiotics which caused negative results (Saukkoriipi *et al*., 2004; Takahashi and Nakayama, 2006; Sacchi *et al*., 2011).

From the results above it may suggest that small amount of bacteria present in samples can be detected by PCR assay than culture. Saukkoriipi *et al*. (2004) noted that PCR assay may detect dead bacteria or bacteria with impaired viability present in clinical samples which may cause discrepancies between the PCR and culture results.

In conclusion the developed TaqMan real time PCR is suitable for identification and quantification of *H. influenza*e type b in CSF specimens which will be helpful in diagnosis, and treatment in future.

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