IJBPAS, September, 2017, 6(9): 1648-1661

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

ANALYTICAL SPECIFICITY AND SENSITIVITY DETERMINATION OF THE *OMPP6* GENE WITH DEVELOPMENT OF INTERNAL POSITIVE CONTROL (IPC) BASED ON TAQMAN TECHNOLOGY FOR IDENTIFICATION OF *HAEMOPHILUS INFLUENZAE* BACTERIUM

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ABSTRACT

Haemophilus influenzae meningitis, a bacterial infection due to the type b (Hib) of this agent, is the main reason formortality and morbidity in children and adults. In the recent years, molecular diagnosis of *H.influenzae* has been carried out by various genes of the mentioned agent. In this study, *ompp6* gene was targeted and the analytical specificity and sensitivity of the indicated gene were determined using Taqman Real Time PCR. In addition, to accurate interpretation of PCR results, internal positive control (IPC) based on the *ompp6* gene with a novel strategy was developed. Evaluation of the assay specificity demonstrated that on the basis of the *ompp6* gene it's possible to discriminate between *Haemophilus influenza* and bacterial agents especially other bacterial meningitis agents such as *Neisseria meningitidis* and *Streptococcus pneumoniae*. Sensitivity and the limit of detection (LOD) of the *ompp6* gene for detection of *H. Influenzae* acquired 1 pg. Amplification of IPC in the presence of target gene exhibited simultaneous

amplification of IPC and target gene in the same reaction tube. The results of the current study concluded that the *ompp6* gene is a highly specific and sensitive gene for molecular detection of *Haemophilus influenzae* bacterium.

Keywords: *Haemophilus influenzae, ompp 6* gene, meningitis, Taqman Real-time PCR, analytical specificity and sensitivity, internal positive control

INTRODUCTION

Haemophilusin fluenzae causes wide variety of infections ranging from respiratory tract infections to meningitis in children and adults. Theylive symbiotically in the upper respiratory tracts of humans (Van Eldere et al., 2014). *Haemophilus influenzae* type b (Hib) recognized as a main pathogenic although is only found in around 7% of the population and is a major cause of meningitis resulting in important mortality and morbidity in children less than five years in all societies worldwide (Briere et al., 2014). For this reason rapid and the exact etiologic diagnosis of bacterial meningitis is crucial. Two important diagnostic tests for detection of bacterial meningitis are gram stain and culture. Previous studies showed that gram stain is fast but unspecific and has a low sensitivity. On the other hand culture take up to 36 hours or more; meanwhile, sometimes due to antibiotic therapy beforehand, bacteria cannot grow on culture media and may show negative results (Wu et al., 2013; Dodémont et al., 2014). Several other laboratory methods based on bacterial antigen, such as latex agglutination test and counter-current immunoelectrophoresis for specific and rapid detection of *Haemophilus influenzae* have been progressed (Mohammadi et al., 2013). Although these methods are specific and faster than culture or gram stain tests but have a lower sensitivity and also false-positive results may happen. Consequently, in the recent years molecular diagnostic methods such as PCR and Real Time PCR have been developed to detection of *H. influenza* bacterium based on different genes of this agent. For example *bexA*, *16SrRNA*, *hpd*, *hel* and the *ompp6* genes have been employed for PCR detection of this agent (Selva et al., 2013; Wang et al., 2014;Gadsby et al., 2015). Among these genes, the *ompp6* gene is the best candidate for molecular detection of *H.influenzae* because the *ompp6* gene is highly conserved and exist in the all capsulate and non-capsulate strains of this bacterium and in conformity with this gene be able to distinguish between pathogen and non patogen strains (Abdeldaim et al., 2013; Price et al., 2015; Mihret et al., 2016). On the

othe side, recent studies have shown that although polymerase chain reaction (PCR) method is a useful diagnostic tool for detecting infectious agents, but false negative results can also occur due to the existence of PCR inhibitor or error in the reaction ingredients (Selva et al., 2013; Hammitt et al., 2014; Van Eldere et al., 2014; Price et al., 2015). To overcome these problems, the use of an internal positive control in diagnostic test has been recommended (Diaz et al., 2013; Jartti et al., 2013; Wu et al., 2013). Accordingly, in the current study, analytical specificity and sensitivity of *Haemophilus influenza ompp6* gene were determined for rapid detection of *H. Influenzae* using Taqman Real Time PCR and an Internal Positive Control (IPC) for this gene was developed.

MATERIALS AND METHODS

Microorganism strains and Plasmid

Fourteen lyophilized stock bacterial strains were kindly provided byPasture Institute of Iran (Tehran, Iran) and were revived in brain heart infusion broth (BHI) medium (Table 1). DNA extraction from bacterial agents was done by DNA Extraction kit (Cinnagen Company, Tehran, Iran) according to manufacturer's instructions. These genomic DNA were used for determination of the assay specificity. *Pichia pastoris* strain *GS115*was purchased from Invitrogen Corporation (Carlsbad, CA, USA) was used for construction of Internal Positive Control (IPC). Isolation of total DNA from yeast was performed as described by Lin-Feng You *et al* (You et al., 2014). Also in this study pTZ57R/T-ompp6 plasmid (containing 280bp of *Haemophilus influenza* bacterium *ompp6* gene) was selected, which had been created in previous study of this paper authors.

Primers and Probes design

Primer design was carried out on the *ompp6* gene of *Haemophilus influenzae*. For this purpose, all submitted sequences of the gene were acquired from Genebank, NCBI **(AAZE01000002, AAZG01000003, ABWV01000002, NZ_AAZE01000002, NZ_AAZG01000003, NZ_ABWV01000002, CP000057, NC-007146, AAZD01000002, NZ_AAZD01000002, AAZF01000002, ABWW01000002, NZ_AAZF01000002, NZ_ABWW01000002, AAZH01000002, AAZI01000002, ACSL01000005, CP000671, CP002276, CP002277, NC_009566, NZ_AAZH01000002, NZ_AAZI01000002, NZ_ACSL01000005, ACSM01000003, NC_000907, NZ_ACSM01000003, FQ312006, AAZJ01000001, NZ_AAZJ01000001, HM124553, HM124552, HM124551)**. The sequences were aligned by CLC sequence viewer software v6 (CLC bio, Aarhus, Denmark). Agreeing to highly conserved regions of *ompp6* gene, the primers and Taqman probe accomplished by Allele ID software v7 **(**PREMIER Biosoft**,** CA, USA) were designed (F-ompp6, R-

ompp6, P-ompp6). Specificity evaluation of the primers and probe were executed with primer-BLAST online software (**http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome**).

In order to IPC construction, a kind of hybrid primers were designed (F-AOX_1 and R-AOX_1) that had the 5′-endsthe same as full sequence of the *ompp6* forward and reverse primers sequence but 13 and 12 nucleotide of the yeast *Pichia pastoris* AOX_1 gene existed in the 3′-ends of forward and reverse hybrid primers, respectively. In silico PCR with the said primers in genomic DNA of *Pichia pastoris* was generated the desired new heterologous sequence. Regarding the IPC amplification with Taqman technology, another probe was designed (P-IPC) by Allele ID software v7 **(**PREMIER Biosoft, CA, USA) on the basis of introduced sequence, F-ompp6 and R-ompp6primers. The 5′-end of the *ompp6* probe labeled with 6.carboxy- N, N, N′, N′-tetramethylrhodamin (TAMRA) and the 3′-end-labeled with Black Hole Quencher-2 (BHQ_2) as the reporter and quencher fluorescent dyes, respectively. On the other side 5′-end of IPC probe labeled with 6-Hexachlorofluorescein (HEX) dye and 3′-end labeled with Black Hole Quencher-1 (BHQ_1) dye. Primers and probes synthesis were performed by Bioneer Corporation (Seoul, Korea). The primers and probes sequences are listed in table 2.

Taqman Real time PCR setup

Taqman Real time PCR assay was conducted with 7500 Fast Real Time PCR system (Applied Biosystems, USA). The reaction volume was 20 µl, containing: 10 µl of premix ExTaq Buffer (2x) (TaKaRa, Japan), 0.4 µl of each F-ompp6 and R-ompp6 primers (10µM), 0.8 µl of P-ompp6 probe (10 µM), 0.4 µl of ROX (50X), 6 µl of nuclease free water and 2 µl of pTZ57R/T-ompp6 (10 ng). The instrument was run with following thermal condition: initial denaturation at 95˚C (5 min), 40 cycles consist of: denaturation at 95˚C (5 sec), annealing and extension at 60˚C (30 sec). Eventually, generated fluorescent signal related to degradation of Taqmanprobe during amplification reaction was calculated using the ABI 7500 software v2.0.1 (Applied Biosystems, CA, USA)

Analytical specificity determination

Genomic DNA of fourteen bacterial strains (Table 1) was applied for analytical specificity determination of the assay. These genomic DNA were amplified with specific primers and probe of *Haemophilus influenza* Bacterium.

Analytical sensitivity determination and generation of standard curve

In sensitivity examination, the assay Limit of Detection (LOD) was determined. For this purpose at first, the concentration of pTZ57R/T-ompp6 plasmid was calculated using the spectrophotometric measurement of the absorption at 260 nm (Picodrop Limited, Saffron Walden, UK). Then, 10 fold serial dilutions of pTZ57R/T-ompp6 plasmid were prepared (10ng-1fg). Taqman Real time PCR was performed in triplicate on standard serial dilutions. In accord with standard dilutions data, the standard curve was design by plotting the DNA amount against the C_t value exported from instrument. The standard curve concluded the coefficient (R^2 value) and reaction efficiency.

The assay validation

In order to the assay validation, CSF was contaminated with various concentration of pTZ57R/T-ompp6 plasmid (10ng-1pg) the same as standard serial dilutions which prepared for analytical sensitivity determination test. Plasmids were isolated from all specimens through QIAamp^R DNA Mini Kit (QIAGEN, CA, USA) following manufacturer's instruction. Finally, all isolated DNA were subjected to Taqman Real Time PCR. Also, the concentrations of 10ng-1pgof pTZ57R/T-ompp6 plasmid were amplified directly in Taqman Real Time PCR.

Construction of Internal Positive Control (IPC)

PCR reaction was carried outin 25 µl volumes comprising of: 10X PCR buffer, 1u/µl Taq DNA polymerase, 1.5 mM $MgCl_2$ (Kowsar, Tehran, Iran), 0.2 mM $dNTP_S$ (Fermentas, USA), 10 µM from each F-IPC and R-IPC primers, 10 ng from *Pichia pastoris* genomic DNA. The PCR were run atthe annealing temperature of 35˚C. Also one reaction was prepared as PCR negative control with deionized water instead of DNA. The PCR products were visualized by staining with ethidiumbromide. After that the PCR product was purified with PCR purification kit (Bionner, Seoul, Korea). The purified PCR product was ligated into the pTZ57R/T vector and then was cloned into *E.coli*JM107 through Ins TA clone^{TM} PCR Cloning kit (Fermentas, USA) according to the manufacturer's instruction. The plasmid was extracted from the recombinant*E.coli*JM107by ACCU Prep plasmid mini extraction kit (Bionner, Seoul, Korea). The presence of heterologous sequence in pTZ57R/T vector was confirmed by sequencing. This plasmid was named pTZ57R/T-IPC.

Co-amplification of pTZ57R/T-ompp6 and pTZ57R/T-IPC

Multiplex Taqman Real time PCR reaction was developed with the premix Ex Taq Buffer (2x) (TaKaRa, Japan) in 20 µl volume a same as above condition go along with 0.8 µl of P-ompp6and P-IPC probes (10µM) accompanied by 2 µl of pTZ57R/T-ompp6 and pTZ57R/T-IPC (50ng) as the DNA templates.

Determining of IPC optimal concentration

In this process, the concentration of pTZ57R/T-IPC was calculated using a picodrop spectrophotometer at 260 nm (Picodrop Limited, Saffron Walden, UK). Then 10-fold standard serial dilutions (3ng – 3fg) were prepared from pTZ57R/T-IPC plasmid. Twice Multiplex Taqman Real time PCR in triplicate was performed on all standard serial dilutions goes along with the lowest detectable concentration of the *ompp6* gene.

RESULTS AND DISCUSSION

Analytical specificity determination

Amplification growth curve related to TAMRA dye showed specific amplification of the *ompp6* gene by designed primers and probe, while no amplification signal was observed with Taqman Real Time PCR on genomic DNA of other bacterial agents through specific primers of *Haemophilus influenza ompp6* gene. In order to evaluate the negative amplification in the analytical specificity determination test and the effect of PCR inhibitors on test results, the *16SrRNA* genes of these agents were targeted for amplification with the universal primers (Yarza et al., 2014). Positive amplification of negative control bacterial *16SrRNA* genes with universal primers demonstrated that these genomic DNA were PCR-able. Consequently, the results of analytical specificity determination of the *ompp6* gene exhibited that the *ompp6* gene is a highly specific gene for molecular detection and differentiation between *Haemophilus influenzae* and other bacterial agent's especially bacterial menangitiditis agents.

Analytical sensitivity determination

A variety of methods exist for analytical sensitivity determination. Some researchers used CFU method for estimating the limit of detection. In this method, after preparation of serial dilutions of the bacterial culture and the optimum incubation temperature, then all serials were used for analytical sensitivity determination. Maaroufi et al. (2007) engaged CFU method for sensitivity determination. They estimated the LOD of their assay 10^1 CFU/PCR, 10^3 CFU/PCR and 10^4 CFU/PCR for types a, b and c, type e and type d and f, respectively (Maaroufi et al.,

2007).Time consuming and the need to the bacteria alive, are the restrictions of the mentioned method. Another method is preparation of serial dilutions from bacteria genomic DNA. Wroblewski et al. (2013) used genomic DNA of *Heamophilus influenzae* for sensitivity determination of PCR assay together with p6 primers. They determined the limit of detection of their assay 10pg (Wroblewski et al., 2013). Billal et al. (2007) to detection of *H. influenza* estimated the LOD of multiplex PCR assay 2pg (Billal et al., 2007). Studies illustrated that the genomic DNA because of largeness is very sensitive to temperature conditions and is damaged easily. In the current study to overcome these problems, *ompp6* gene PCR product was ligated into the pTZ57R/T plasmid and the resulting construct was cloned in *E.coli JM107*. Due to the smaller size of plasmid than genomic DNA, this procedure can cause stability of gene target against to environmental conditions. Also plasmid DNA versus genomic DNA because of its smaller size, during preparation of serial dilutions will be allocated equally. Hence this plasmid is becoming option to use as the standard positive control. In the present study for analytical sensitivity determination, serial dilutions were prepared from standard positive control plasmid then all serials were subjected to Taqman Real Time PCR using the *ompp6* specific primers. As shown in figure 1A, the last concentration of positive control plasmid which had the amplification signal, was 1pg. Therefore the limit of detection of the assay determined as 1pg. According to the threshold cycles (C_T) of amplification growth curve related to standard serial dilutions (Y axis) against the concentration of each fold (X axis), standard curve was design (figure 1B). The slope of the curve was -3.498, efficiency was 93.142 and the R^2 value was 0.999. Reproducibility of the assay was twice examined. The results of two exams were similar. These tests confirmed the precision of the assay (Huang et al., 2013).

A clinical sample for detection of *H. influenzae* is cerebrospinal fluid (CSF). In this study, CSF was selected for assay validation using artificial contamination technique (Wu et al., 2013; Zhao et al., 2014).Giving to table 3, for evaluation of the accuracy in the assay validation, four individual Taqman Real Time PCR were carried out. In the first and second experiments, concentrations of 10ng-1pg of pTZ57R/T-ompp6 (The same as analytical sensitivity determination test) were utilized directly in the assay but in the third and fourth experiments, the indicated

concentrations were engaged for artificial contamination of CSF. Afterwards, isolated DNA of these CSF samples was subjected to amplification by Taqman Real Time PCR. In agreement with table 4, comparison of experiments 1 and 2 with 3 and 4 showed when isolated DNA of CSF were employed in the Taqman assay, the C_T value of each concentration without any effect in assay sensitivity, slightly increased. These test verified the accuracy of the assay for the detection of *H.influenzae* in clinical specimens. Since all the concentrations of positive control plasmid after extractions of CSF were still able to amplify, as a result, this plasmid can be used in the extraction positive control in order to examine and check of the DNA isolation procedure from CSF.

Construction of IPC

Although the positive control plasmid is a control sample which was used in the assay but could not demonstrate the PCR ability for the test sample. In other words, false negative results may occur in the test sample on account of instrument failure or the presence of PCR inhibitor in clinical specimens (Santiago et al., 2013). Hence, the use of the internal positive control is necessary and recommended for exact interpretation of PCR results (Majidzadeh et al., 2014). Two types of IPC exist, noncompetitive IPC and competitive IPC as suggested that competitive IPC is better than noncompetitive form because in the noncompetitive form the existence of two primer pairs in a single reaction tube may lead to contribute to low sensitivity of the assay (Wang et al., 2015). In recent years, the various methods for construction of competitive IPC have been reported (Ling et al., 2014; Vanysacker et al., 2014). In the current study, the approach used for construction of IPC was to create a new heterologous sequence which does not have any homology with bacterial sequences especially the *ompp6* sequence. For this objective, *AOX1* gene of the yeast *Pichia pastoris* was candidate because *AOX1* gene was a eukaryotic gene and did not any proximity to *ompp6* prokaryotic gene. Amplification of *Pichia pastoris* genomic DNA through PCR with hybrid primers, displayed the predicted 260bpb and. The nucleotide sequences of 5′ and 3′ ends of the resulted product were the same as the diagnostic primers nucleotide sequences of the *ompp6* gene, but in the middle of the mentioned product, *AOX1* gene sequence of *Pichia pastoris* existed. The purified PCR product cloned into pTZ57R/T vector. Sequencing results of the recombinant

plasmid with M13 primers demonstrated the presence of the IPC sequence in T- vector. Simultaneous amplification of IPC and target gene in the same reaction tube with two probes revealed concurrent presence of two different growth curves related to fluorescence signal of TAMRA and HEX dyes in different channel. These data confirmed the simultaneous amplification of pTZ57R/T-ompp6 and pTZ57R/T-IPC plasmids with the same primers and different probes.

Since constructed IPC in this study was kind of competitive IPC, to prevent the inhibitory effect of IPC in target gene amplification, the minimum concentration of amplifiable IPC was determined as IPC optimal concentration. In order to determine the IPC optimal concentration, pTZ57R/T-IPC in the presence of pTZ57R/T-ompp6 (LOD concentration, 1pg) co-amplified twice using multiplex Taqman Real time PCR in triplicate. The C_T value for both IPC and *ompp6* genes were calculated by 7500 software (version 2.01). The mean of the C_T value for each concentration of every experiment is represented in table 4. As illustrated in table 4, in both experiments, amplification signal observed in 3pg concentration of IPC while amplification signal related to 300fg concentration of IPC is only observed in the first experiment. Corresponding to these data, the best concentration of IPC for using in the assay in the presence of the *ompp6* gene, the LOD was determined as 3pg.

Table 1: The list of negative control bacterial was used for analytical specificity determination of the assay

Microorganism name	Strain Number
Shigellasonnei	ATCC 9290
Klebsiella pneumoniae	ATCC 7881
Escherichia coli	ATCC 25922
Bacillus subtilis	ATCC 6051
Staphylococcus aureus	ATCC 25923
Enterococcus faecalis	ATCC 29212
Salmonella Typhi	ATCC 700931
Streptococcus penumoniea	ATCC 700669
Neisseria meningitidis	ATCC13060
E.coli O157H7	ATCC 43895
E.coli EPEC	ATCC 43887
Yersinia enterocolitica	PTCC 1480
Yersinia pseudotuberculosis	ATCC 29833

Table 2: The list of primers and probes were used in this study

name	sequences	product size
F-ompp6	ACTGGTGAATACGTTCAAATCTTAG	182 bp
R-ompp6	CCAGCATCAACACCTTTACC	
P-ompp6	ACCTTTAACTGCATCTGCACGACGTTG	
F-AOX_1	ACTGGTGAATACGTTCAAATCTTAGAGCAGACCGTTGC	260 bp
R-AOX_1	CCAGCATCAACACCTTTACCATGCGGAGCTTG	
P-IPC	ACCTCCACTCCTCTTCTCCTCAACACC	

Table 3: The C_T value of various concentrations of pTZ57R/T-ompp6 for evaluation of the assay validation

Concentrations	Repeat	Exp 1	Exp 2	Exp 3	Exp 4
10ng	1	23.55	23.42	31.30	31.79
	2	23.53	23.33	30.45	31.73
	3	23.17	23.14	31.55	31.92
1ng	1	27.25	27.45	32.16	33.65
	2	27.06	27.14	33.43	33.76
	3	26.97	27.78	32.56	34.10
100pg	1	30.71	31.61	34.24	34.14
	2	31.14	31.71	34.72	34.49
	3	30.53	30.48	34.54	34.44
10pg	1	34.53	33.57	34.63	33.84
	2	34.39	33.64	34.14	34.85
	3	34.58	33.58	33.65	33.98
1pg	1	37.31	37.71	37.30	36.44
	2	38.21	38.11	36.39	36.90
	3	37.97	37.28	36.14	36.88

Table 4: The C_T value related to amplification of IPC and *ompp6* genes

IPC gene concentrations / *Ompp6* gene LOD Concentration	CT value of IPC/ *ompp6* genes in the first experiment	CT value of IPC/ *ompp6* genes in the second experiment
3ng/1pg	18.08/undetermined	18.34/undetermined
300pg/1pg	22.06/undetermined	22.22/undetermined
30pg/1pg	25.83/undetermined	27.08/undetermined
3pg/1pg	29.85/35.54	33.81/32.94
300fg/1pg	33.21/33.86	Undetermined/33.74
30fg/1pg	Undetermined/35	Undetermined/32.2
3fg/1pg	Undetermined/34.9	Undetermined/35

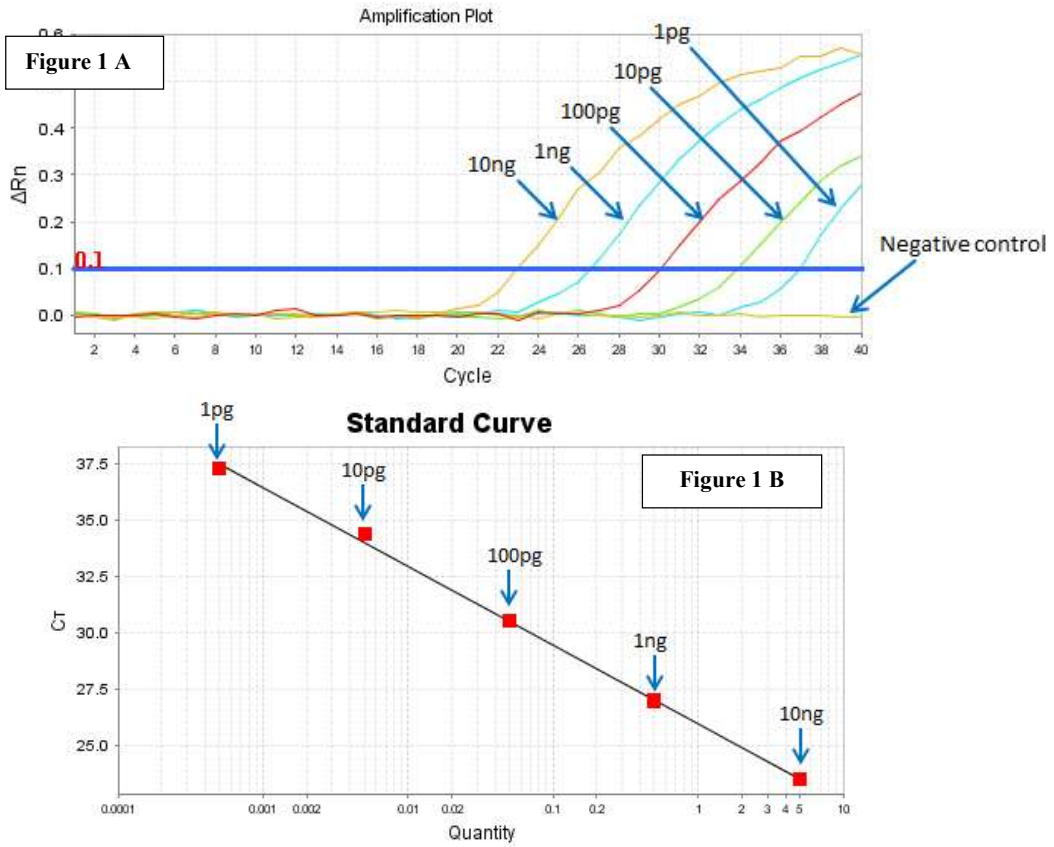

Figure 1 A: The Amplification plot related to standard serial dilutions series. According to this figure, the LOD of assay was 1pg. B: The Standard curve corresponding to standard serial dilutions of *ompp6* gene. As seen in the figure, the slope was -3.498, R^2 was 0.999 and Eff% was 93.142

CONCLUSION

In Conclusion, the competitive IPC can be used in both target and control reaction tubes, with same primers and so there were no important alterations of the amplification procedures. The results of this study recommended that the Taqman Real time PCR amplification assay in the presence of the internal positive control could provide an accurate diagnosis of Haemophilus influenzae infection.

ACKNOWLEDGMENTS

The authors would like to acknowledge and appreciate the Faculty of Medicine, AJA University of Medical sciences and Tasnim Biotechnology Research Center (TBRC), for their support and contribution to this study.

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