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A Molecular and Comparative Study of Type-able and Non-type-able *Haemophilus influenzae* isolated from different clinical samples in Hilla, Iraq.

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Abstract

Background *H. influenzae* bacteria classified as type-able and non-type-able according to the presence or absence of capsule which is correlated with site of isolation.

Objectives To isolate *H. influenzae* from different clinical samples and differentiate both capsulated (type-able) and non-capsulated (non-type-able) one by molecular detection method and to make a comparison between the two types by cultural, molecular, and clinical aspects.

Methods A total of 220 clinical samples were aseptically taken from throat, ear, eye, sputum and CSF of patients attended three main hospitals in Hilla city, Iraq during the period from Feb. 2012 to Jun. 2012. All samples were subjected to bacterial cultivation, standard bacteriological method and molecular detection method. Other primers were used to detect the presence or absence of capsule using Bex A, Bex B, while p2 primer was used to detect non-type-able. Among capsulated one a specific primers (Hib, bex) targeting *H. influenzae* type b were used.

Results 29 (13.2%) out of 220 clinical samples give presumptive detection and isolation of *H. influenzae*, of these only 10 (34.5%) were positive using X (haemin) and V (Nad) tests. Using PCR, only 6 out of 10 (60%) were positive, out of 6 isolates only 2 (33.3%) were capsulated (type-able) and 4(66.6%) non-type-able, out of 2 capsulated 1 (50%) was type b *H. influenzae*.

Conclusion Specific genetic marker should be used to detect both types. Many non-type-able *H. influenzae* isolates are also important cause of upper respiratory tract diseases including pharangitis, otitis media, and conjunctivitis. Using serotype specific gene targeting type b (Hib) is important among patient with meningitis and lastly separation between type-able and non-type-able according to the site of isolation.

Keyword *H. influenzae* , genetic marker, serotype specific gene, Hib, Bex(A, B) P6, NTHI, X,V factors

Introduction

Haemophilus influenzae (*H. influenzae*) is a Gram negative coccobacillus whose environmental niche is primarily restricted to the human respiratory tract. The genus name reflects its absolute requirement for heme and the species name reflects the erroneous initial belief that this organism was the causative agent of influenzae [(1)]. Generally, it is difficult and time-consuming to detect *H. influenzae* by using traditional method. So, PCR-based method targeting the outer membrane protein (OMP) represents specific diagnostic targets. OMP P6 is highly conserved among most strains of *H. influenzae* [(2)]. It is a peptidoglycan-associated lipoprotein constitutes 1-5% of all OMPs. Unlike P2 protein, P6 protein shown a very high homology (97%) in the amino acid

analyses of type b and non-type-able *H.influenzae,* which shows that this protein is stable and conserved [3]. Other OMP is P2 which is a major protein comprises more than 50% of the OMPs. This protein exists on the outer membrane as a trimer and act as a prion, so, it is mostly located on the outer membrane of non-type-able *H. influenzae* [4].

H. influenzae is also variable for the presence of polysaccharide capsule and is classified on the basis of production of polysaccharide capsule, strain types a through f produce antigenically distinct capsules and non-type-able strains produce no capsule [5]. Others classify *H. influenzae* into 3 main categories: nonencapsulated strains, encapsulated type b strains, and capsulated non-type b strains (types a and c-f) where type b is the most virulent form [6]. Several studies have demonstrated molecular capsule typing methods to be more sensitive and specific than other methods [7].

Encapsulated *H. influenzae* isolates contain genes for the production of their respective polysaccharides capsules at the cap locus which is composed of three distinct regions, designated region I to III. The genes contained within regions I and III, designated bex DCBA and hcs AB, respectively, are highly conserved across all six capsular types and are required for transport of capsule constituents across the outer membrane [8,9]. Yet, region II gene encodes capsule type a-through f-specific proteins and thus varies by serotype.

The organization and genetics of the cap locus are complex, duplications, partial loss, and complete loss of the cap locus can occur and give different results [10]. Early studies revealed that all isolates of *H. influenzae* are different in terms of pathogenic potential. It is very important to mention that most systemic isolates express the type b capsule, whereas most respiratory tract isolates contain unencapsulated, referred to as non-type-able [11]. These are more commonly part of the normal flora, less invasive and frequently involved in opportunistic respiratory tract infections. Many classification approaches showed that most non-type-able *H. influenzae* (NTH1) were genetically quite distinct from type b (Hib) strains and more heterogenous, yet, ribotyping and ERIC (Enterobacterial Repetitive Intergenic Consensus), PCR have been used to relate strains. The horizontal exchange of *H. influenzae* genetic loci between strains due to natural DNA transformation make the classification complicated and may explain the differences seen using different methods [12].

The objectives of this study was to isolate *H. influenzae* from different clinical samples and differentiate both capsulated (type-able) and non-capsulated (non-type-able) one by molecular detection method and to make a comparison between the two types by cultural, molecular, and clinical aspects.

Methods

Samples and Bacterial culture

A total of 220 clinical samples were taken from different clinical samples from patients attending the three main hospitals (Babylon Hospital for Maternal and Pediatrics, Al-Hilla Surgical Teaching Hospital and Merjan Medical City) during the period from February 2012-June 2012. The samples were transported using specific transport media and processed on blood, chocolate agar and tryptic Soya agar sublimated with X, V disc and subjected to standard bacteriological method and incubated in 5% CO_2 at 37^{o}C for 24 hrs., biochemical tests like catalase, oxidase, urease, indole, nitrate reduction, carbohydrate fermentation was done according to MacFaddin [13].

DNA extraction from Gram-negative bacteria

This method was performed according to the genomic DNA purification kit supplemented by a manufacturing company (Promega, USA).

Molecular method used in detection of type-able and non-type-able *H.influenzae*

H. influenzae was detected by PCR, using 20 µl PCR reaction mixture as in table 1. Primer used and thermal cycle condition were illustrated in table 2. The amplification product was separated on (1-1.5%) agarose gel containing ethidium bromide for 45 min. at 70 V. The size of the

amplicons was determined by comparison to the 100 bp allelic ladder (Promega, USA).

Table 1. Contents of reaction mixture

No.	Contents of reaction mixture	Volume (µl)
1	Green master mix	5
2	Upstream primer	3
3	Downstream primer	3
4	DNA template	5
5	Nuclease free-water	4
Total volume		20

Table 2. Primers sequences and thermal cycler conditions

Genes	Primer sequence (5'-3')	Size of product bp	PCR condition
P6F **P6R**	5-AACTTTTGGCGGTTACTCTG-3 5-CTAACACTGCACGACGGTTT-3	351	95 °C 10 min 1X 95 °C 30 sec 55 °C 1 min 30X 72 °C 2 min 72 °C 5 min 1X 95 °C 10 min 1X [31]
P2F **P2R**	5-GTTCACGTTTCCACATTAAAGC-3 5-CACGACCAAGTTTTACTTCAC-3	186	95 °C 30 sec 55 °C 1 min 35X 72 °C 2 min 72 °C 5 min [32]
Bex A F **Bex A R**	5-CGTTTGTATGATGTTGATCCAGAC-3 5-TGTCCATGTCTTCAAAATGATG-3	343	95 °C 2 min 1X 95 °C 30 sec 54 °C 30 sec 30X 72 °C 45 sec 72 °C 5 min 1X [33]
Bex B F **Bex B R**	5-GGTGATTAACGCGTTGCTTATGCG 5-TTGTGCCTGTGCTGGAAGGTTATG	567	95 °C 2 min 1X 95 °C 30 sec 54 °C 30 sec 30X 72 °C 45 sec 72 °C 5 min 1X [21]
Hib F **Hib R**	5-CCTCGCAATGCAGTTTATGGTCC-3 5-AAGCGGGAATTTGATACCTGATGC-3	774	94 °C 3 min 1X 94 °C 30 sec 60 °C 1 min 37X 72 °C 40 sec 72 °C 5 min 72 °C 1 min [34]
Bex F **Bex R**	5-TATCACACAAATAGCGGTTGG-3 GGCCAAGAGATACTCATAGAACGTT-3	81	95 °C 5 min 1X 95 °C 25 sec 57 °C 40 sec 35X 72 °C 10 min 1X [35]

Results

Among the 220 clinical samples only 29 (13.2%) isolates gave presumptive detection of *H. influenzae* and out of these 29 isolates only 10 were positive by using X (haemin) and V (NAD) tests that are required for growth of *H. influenzae* and these isolates were distributed and isolated from different clinical samples mainly throat, ear, eye, sputum, CSF. The 10 isolates then subjected to further molecular detection method using *P6* as a genetic marker for confirmed isolation of *H. influenzae* by PCR as shown in figure 1 and the results revealed that only 6 isolates out of 10 were positive as shown in table 3.

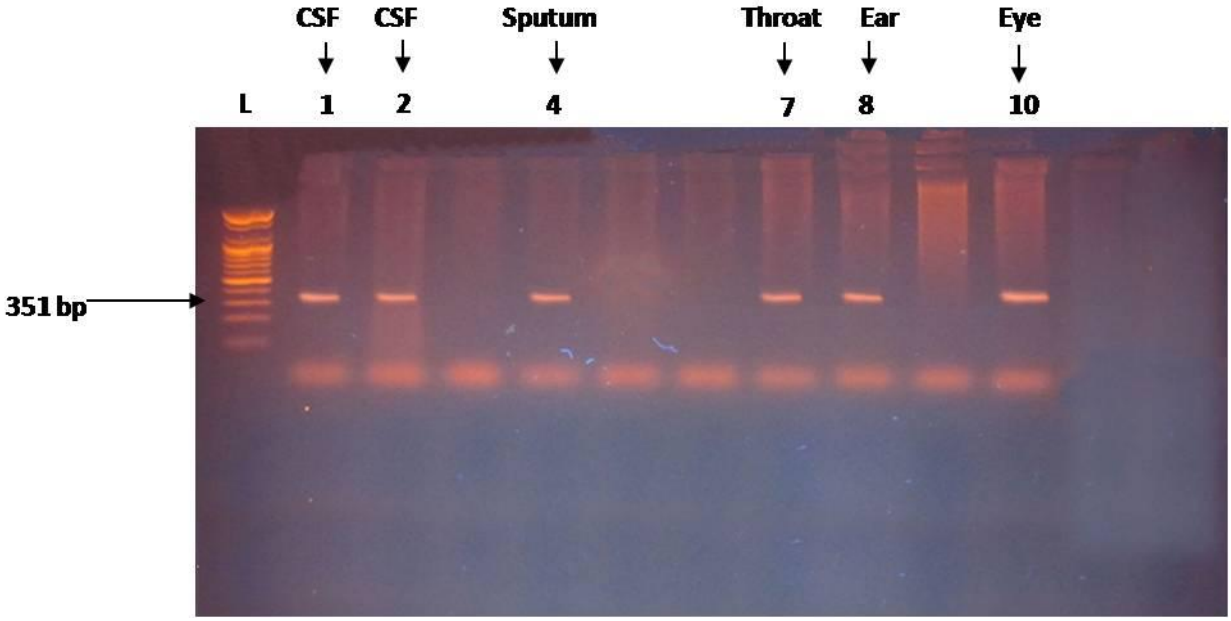

Fig. 1. Gel electrophoresis of PCR product of *P6* gene. Lane of isolates numbered (1,2,4,7,8,10) were positive, where as isolates (3,5,6,9) were negative.

Table 3. Number of isolated *H. influenzae.*

Source of isolates	No. of samples	No. of *H. influenzae* by standard bacteriological method	Using X+V to detect *H. influenzae*	Using PCR by P6 gene
Throat	45	6 (13.3%)	2 (33.3%)	1 (50%)
Ear	50	8 (16%)	1 (12.5%)	1 (100%)
Eye	45	5 (11.1%)	2 (40%)	1 (50%)
Sputum	40	4 (10%)	2 (50%)	1 (50%)
CSF	40	6 (15%)	3 (50%)	2 (66.6%)
Total	220	29 (13.2%)	10 (34.5%)	6 (60%)

All the 6 confirmed isolates of *H. influenzae* underwent testing to separate and differentiate into capsulated (type-able) and non capsulated (non-type-able) depending on the presence or absence of capsule by using specific primers to detect capsule locus of *H.influenzae* namely bexA and bexB. The results revealed that 2 isolates out of 6 (33.3%) were type-able (i.e.,

capsulated) while 4 isolates out of 6 (66.6%) were non-type-able. The results also showed that these 2 capsulated isolates of *H. influenzae* were from CSF of patient with meningitis as shown in figure 2 and 3, respectively.

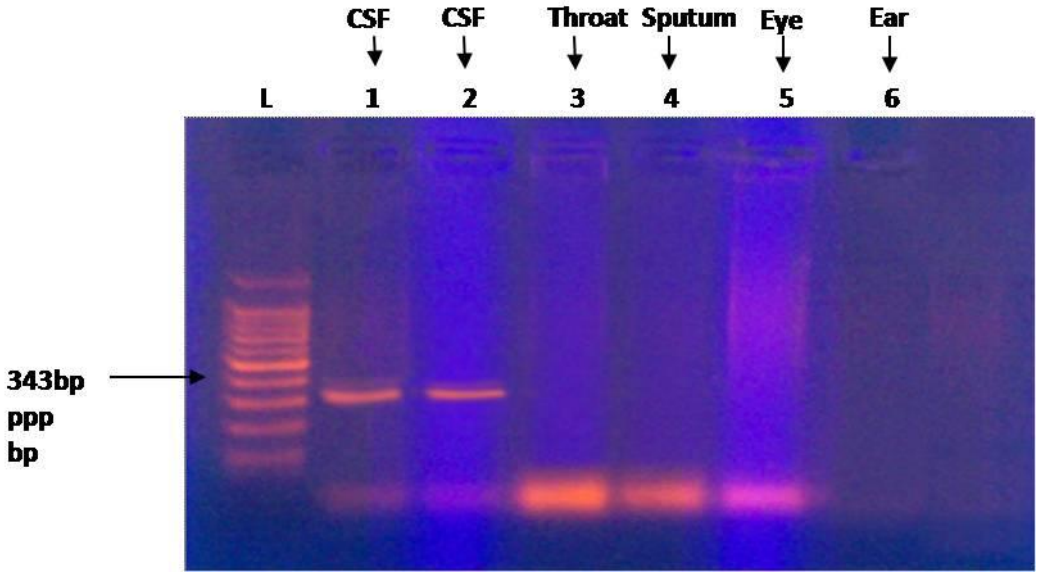

Fig. 2. Gel electrophoresis of PCR product of BexA gene. Lane of isolates numbered (1,2) were positive, where as isolates (3,4,5,6) were negative.

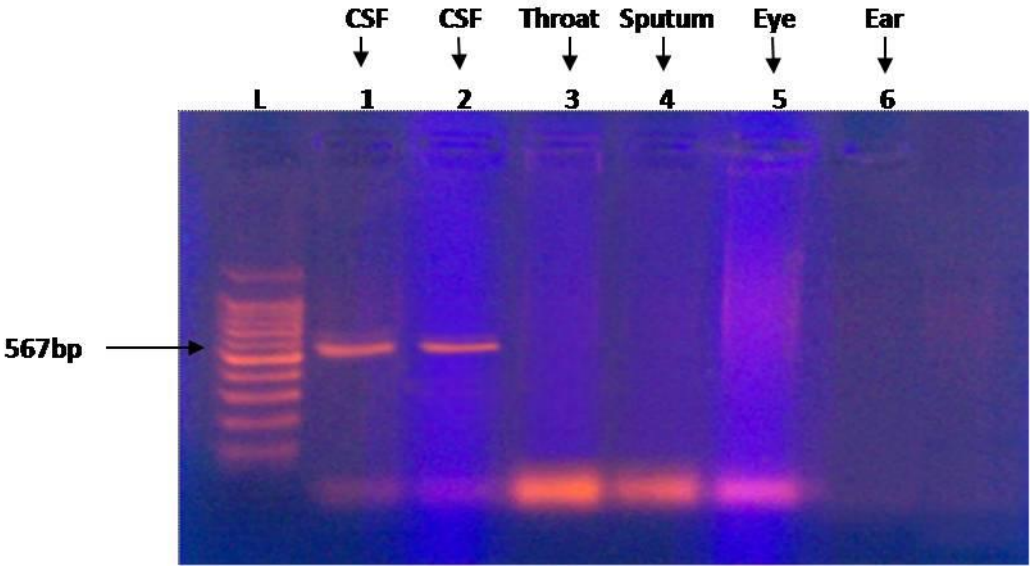

Fig. 3. Gel electrophoresis of PCR product of Bex B gene. Lane of isolates numbered (1,2) were positive, where as isolates (3,4,5,6) were negative.

The remaining 4 isolates 4-6 (66.6 %) give all (100%) positive result for OMP P2 which can be used as a genetic marker for detection of non-type-able one as in figure 4. From the two capsulated one (50%) was of type b H. influenzae by using Hib and bex primers as in figures 5 and 6, respectively.

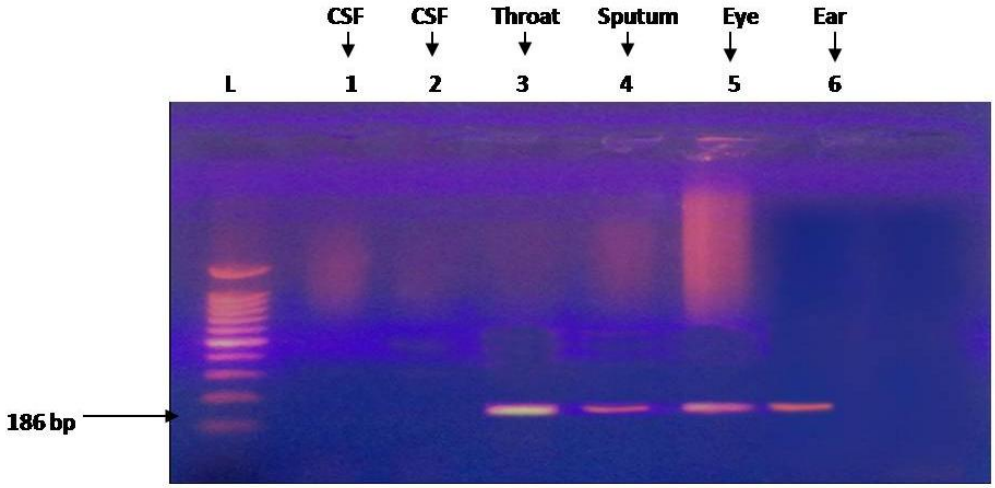

Fig. 4. Gel electrophoresis of PCR product of *P2* gene. Lane of isolates numbered (3,4,5,6) were positive, where as isolates (1,2) were negative.

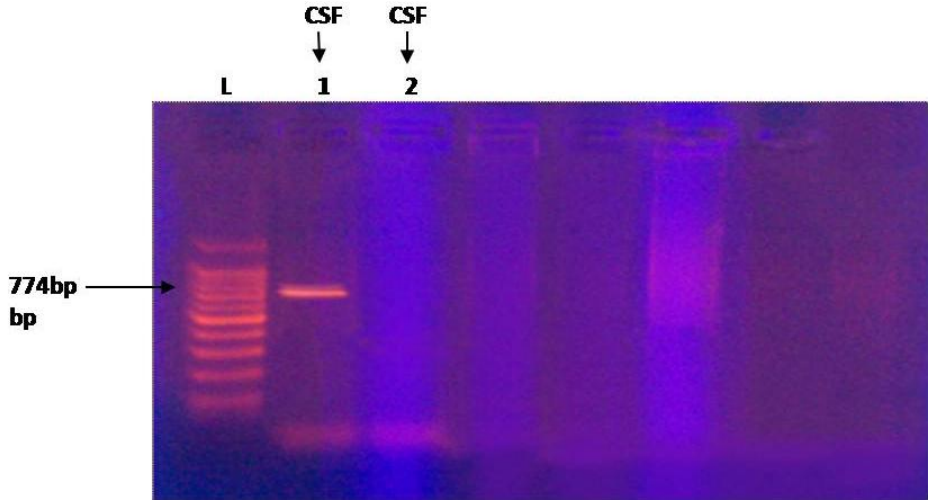

Fig. 5. Gel electrophoresis of PCR product of hib amplicon gene. Lane of isolate numbered (1) was

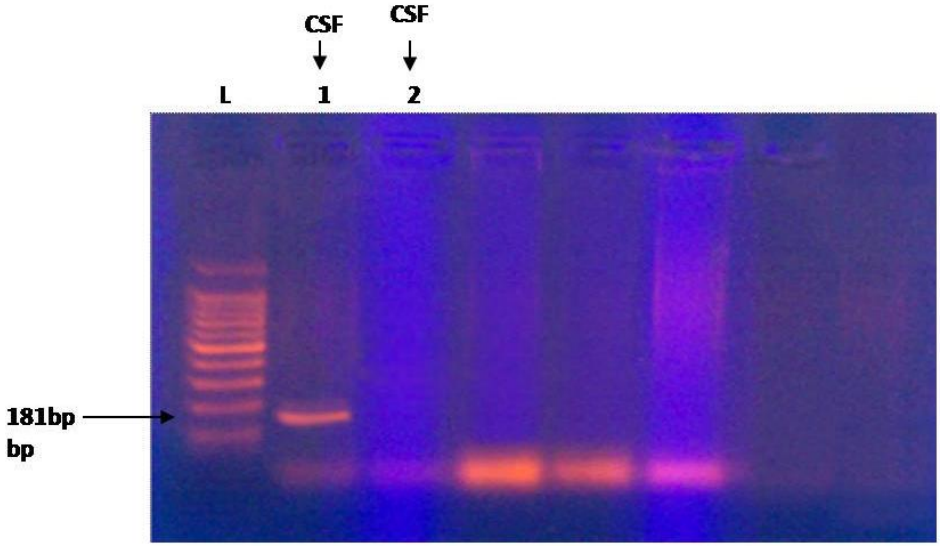

Fig. 6. Gel electrophoresis of PCR product of bex gene. Lane of isolate numbered (1) was positive

Discussion

The results indicate that isolation rate differs according to site of isolation and method applied and this can also be attributed to different virulence factors expressed by *H. influenzae* in different sites of human body as the natural host for it and can be correlated also with the severity and invasiveness of the disease. The results of this study agreed with the results obtained in another study [14] where they detected *H. influenzae* by cultural method and by using X, V discs and specific culture media at a rate of about (18.05%). Also another study found that the isolation of *H. influenzae* is *increased* by using selective media and X, V factors discs and isolated it in a rate of (27.16%) [15]. The same result was described by Mojgani [16] where he isolated *H. influenzae* from CSF of patient with meningitis, eye, mucous from patient with conjunctivitis and nasopharyngeal and ear swabs from patient with otitis media and throat infection, they isolated it at about (31.4%), based on their morphology and growth requirement for X and V factors.

In contrast to the results of this study, other study found 80 isolates of *H. influenzae* on the basis of their growth requirement and serotype distribution [17]. Generally, different isolation rate could also explained by different factors like age, season, the size of the facility, antibiotic treatment, morbidity from acute URIs, the sampling technique ,and the methods that give more accurate sensitivity and specificity. While molecular detection method confirmed the isolation of *H. influenzae* and focused on role of P6 outer membrane protein OMP *P6* gene in this molecular detection method and it detect about 6 isolates (60%) of *H. influenzae* by PCR. Although, *H. influenzae* have many OMPs like P5, P2, but unlik p2 protein, p6 protein show a very high homology (97%) in the amino acid analyses of type b and non-type-able *H. influenzae* strains, which shows that this protein is stable and conserved [3].

Many studies concentrated and focused on this genetic primer and found that *H. influenzae* detection could be achieved with varying degree of success with primers specific for rRNA-encoding genes [18], yet, the rRNA sequences of *H. influenzae* and *H. parainflluenzae* show approximately 95% homology. So, these genes are not ideal targets for the unequivocal identification of *H. influenzae* [19]. Previous studies had shown that NTHi OMP P6 had 100% homology among human respiratory isolates from adults and children [20]. Regarding the determination of capsular type ability by molecular methods detection, the result revealed that 2 isolates out of 6 (33.3%) were type-able (i.e., capsulated) while 4 isolates out of 6 (66.6%) were non-type-able.

The results also showed that these 2 capsulated isolates of *H. influenzae* were from CSF of patient with meningitis and this is accepted regarding the presence of capsule that make it resistant to macrophage, complement and human defense and provides the ability to be invasive and so cause severe disease and even make it resistant to many antibiotics. The highly conserved *bexA* and *bexB* genes, which are required by type-able strain for the transport of capsule components across the outer membrane, were assayed by PCR following the protocol of Davis [21].The advantage of this method over tranditional slides agglutination techniques using type-specific antisera or methods detecting *bexA* alone is that *bexB* PCR will detect rare strains that are *bexA* negative but *bexB* positive, which render them phenotypically non-type-able but genetically far closer to type-able strains [22].

Another studies used PCR reaction to detect *bexA* gene and found that its absence or presence determined whether` an isolate was encapsulated or non capsulated, their study revealed a rate of NTHi to be about 93.5% while type-able one was (6.45%) distributed into different serotypes from a-f [23]. So our results differed from this study and this may be attributed to dependence of their study on *bexA* alone and also their isolates were mostly nasopharyngeal isolates. Many studies found that differentiating type-able from non-type-able *H. influenzae* strains can be challenging

where type-specific serum agglutination had classically been used to confirm the presence and specifity of *H. influenzae* capsule. However, a strain may fail to react with typing sera and thus be classified as non-type-able for several reasons [24].

First: inaccuracy in performing and interpreting slide agglutination tests had been well documented.

Second: strain with one copy of the *cap* region in which *bexA* is partially deleted is referred to as capsule deficient variants because they contain a majority of the *cap* locus.

Third: a previously serotypeable strain could have a deletion of the entire capsule locus, as apparently occurred with strain Rd (non- type-able variant of type d strain).

Fourth and finally: a strain may lack the entire *cap* locus as consequences of long-past evolutionary events, i.e., may be a true NTHi strain [25]. Davis [21] found that *bexB* which is located adjacent to *bexA* in region I of the capsule locus and encodes another protein important in capsule exportation, is a more reliable marker of the capsule locus because it can be detected in *H. influenzae* strains that posses a single *cap* locus and a *bexA* mutation in that locus. Another study done by Mojgani et al [16] isolated *H. influenzae* at a rate different from the rate of this study where NTHi isolation rate was (38.6%). While type-able one was (61%) by using *bexA* primer and found that most of NTHi isolates were from nasopharyngeal secretion while type-able *H. influenzae* were mostly from CSF. The results of this study are in contrast with the results of other studies where they identified and isolated NTHi at a rate of about (33%) while type-able one was (66.6%) but most of their isolates were from invasive *H. influenzae* disease *(26)*. Many studies proposed that the ancestor of *H. influenzae* was encapsulated and the non-type-able clones arose by convergent evolutionary loss of the ability to synthesize or extracellularly express a polysaccharide capsule.

However, the wide heterogenecity of non-type-able strains, the more clonal features of type-able strains and the evidence that most type b specific genetic regions are flanked by repeat sequences and thus may represent acquisition of foreign genetic elements, make it more likely that an un encapsulated ancestral *H. influenzae* strains acquired these elements and became more virulent [27].

While the detection of non-type-able H. *influenzae* at 1st by absence of capsule gene and by P2 gene that is conserved for them. In this study it was used to differentiate between both type-able and NTHi and since it expresses on NTHi so it is possible to confirm that the remaining 4 isolates that was non-capsulated by using Bex primers (A, B) were non-type-able because some isolates that have mutation or deletion or single copy of *cap locus* sometimes fail to express capsule and can be regarding as non- capsulated, but these isolates are not true NTHi.

The emerging role of invasive disease because NTHi is intriguing because this organism had traditionally been considered relatively non-invasive bacteria predominantly associated with community-acquired pneumonia, chronic obstructive pulmonary disease exacerbations and otitis media [28]. Regarding the Detection of type b *H. influenzae*, the study revealed that two isolates were of capsulated (type-able) *H.influenzae and* further primers were used to detect the serotypes of these two isolates and specifically type b (Hib) since these two isolates were from CSF of patient with meningitis and so type b could be the causative agent among these isolates. However, the results showed that only one isolate was type b (Hib) while the other one was capsulated non type b *H. influenzae*.

In this study two primers were used, one called bex to detect Hib from CSF of patient with meningitis specifically and to exclude *N. meningitis* and *S. pneumoniae* while the other one called Hib which confirmed the results and both gave the same results. Center for Disease Control and Prevention [29] revealed that infection with Hib can cause meningitis in 50% of cases of adults and children and this results are similar to the results of this study and they also

revealed to be more prevalent in developing countries. Other serotypes could also be isolated from invasive infection where other studies isolated type a (48%), f (14%) d (5%), c (2%) [26]. Furthermore, the association of non-b-capsular serotypes with invasive disease could be facilitated by the acquisition of virulence factors common to Hib such as capsule gene duplication and an IS1016-bex A deletion in the capsule gene cluster which may serve to stabilize capsule production [25]. In addition, infrequent recombination, event could happen between naturally transformable Hib and other serotype that may enhance the fitness and virulence of these serotypes [30].

We can conclude from the current work that using of specific genetic marker namely P6 primer is important in molecular detection of both types of *H. influenzae* that isolated from different sites. Detection of capsule is valuable in differentiation of type-able from non-type-able one. Non-type-able *H. influenzae* (NTHi) is also an important cause of invasive and severe disease like upper and lower respiratory tract disease and eye infection. Using serotype specific gene is necessary among patient with meningitis since not only type b (Hib) can cause meningitis where other capsulated non b *H. influenzae* could also implicate.

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