

Molecular detection of *Haemophilus influenzae* isolated from eye swabs of patients with conjunctivitis in Hilla Province, Iraq

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ABSTRACT

In this study, 150 eye swabs were collected from patients suffering from conjunctivitis of both sexes admitted to Ophthalmic Unit of Hilla Teaching Hospital during the period from July to December 2020 in Hilla Province, Iraq. These eye swabs were subjected to bacterial culturing, biochemical tests, and Satellitism test using Hemin (factor X) and NAD (factor V) for isolation and identification of *Haemophilus influenzae*. The results showed that 38 (25.3%) out of 150 eye swabs were approved as *H. influenzae* strains and only 26 isolates (68.4%) were positive by using the Satellitism test. These isolates were subjected to PCR technique using *omp6* gene primer for confirming isolation of *H. influenzae*. The results revealed that only 24 (92.3%) out of 26 isolates were positive. Additionally, the PCR technique succeeded in separating these (24) isolates of *H. influenzae* into typeable and nontypeable based on the presence or absence of a capsule by using *bexA* and *bexB* genes primers. The results found that all 24 (100%) isolates did not contain *bexA* and *bexB* genes and are nonencapsulated (nontypeable) isolates.

1. INTRODUCTION

Conjunctivitis is a common type of eye infection usually characterized by inflammation of conjunctivae with swelling of the conjunctival tissue, engorgement of the blood vessels, eye discharge, and irritation. Globally, conjunctivitis affects multiple subjects, and it is one of the important causes of office visits to general medical and ophthalmological clinics [1]. The commonest types of bacteria that cause bacterial conjunctivitis include *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella* spp., *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Proteus mirabilis*, and *Neisseria gonorrhoeae* [2]. *Haemophilus influenzae* is the causative agent and the commonest type of bacteria isolated from patients suffering from bacterial conjunctivitis [3]. It is a Gram-negative coccobacilli bacterium,

and the major virulence factor is the polysaccharide capsule. Depending on the presence or absence of the capsule, *H. influenzae* strains can be subdivided into encapsulated (typeable) and nonencapsulated (nontypeable). Encapsulated strains were divided into six serotypes, ranging from A to F, and comparatively homologous irrespective of their serotype, while the nontypeable strains are genetically diverse [4]. It is a respiratory tract opportunistic pathogen and the largest contributor to invasive disease of *Haemophilus*. During the process of pathogenesis, adhesion plays a major role. Also, they may be diverse in structures like trimeric autotransporters “Hmw1, Hmw2, Hia, and Hap,” surface proteins, “outer membrane proteins,” including OMPs 1, 2, 4, 5, and 6, proteins E, and proteins F, as well as surface protrusions “T4SS and HIF” and the immunogenicity and potential vaccine candidates [5]. Additionally, *H. influenzae* can be classified into three major categories, including (nonencapsulated, capsulated nontype b, and encapsulated type b), where type b is considered as the most virulent form [6].

The objectives of the current study are to isolate and identify *H. influenzae* from eye swabs of patients with conjunctivitis by

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the traditional culture methods, Satellitism test using “X and V” factors, and PCR technique. In addition to differentiating both encapsulated (typeable) and nonencapsulated (nontypeable) *H. influenzae* using *bexA* and *bexB* gene primers by the PCR assay.

2. MATERIALS AND METHODS

2.1. Eye Swabs Collection and Bacterial Identification

The eye swabs were obtained from the conjunctiva of 150 patients with conjunctivitis of both sexes admitted to Ophthalmic Unit of Hilla Teaching Hospital during the period from July to December 2020 in Hilla Province, Babylon Governorate, Iraq. The specimens were transported using specific transport media, and in the laboratory, these swabs were cultured onto the surface of blood agar, MacConkey agar, and chocolate agar. After that, the identified bacterial colonies were subjected to Gram’s stain, and specific biochemical tests such as “catalase, oxidase, Voges–Proskauer, indole, urease, nitrate reduction, and carbohydrate fermentation” were performed to reach the final identification [7].

2.2. Satellitism Test of *H. influenzae*

This test, used to determine *H. influenzae* isolates, was conducted by mixing a loopful of suspected bacterial colonies into approximately 2 ml of sterile saline solution. Inoculate the plates of tryptic soy agar with the bacterial suspension by a sterile swab, and streak a pure culture of *S. aureus* across each plate that had been inoculated. The culture media were then incubated for 18 to 24 hours in 5% CO₂ at 37°C. Observe the suspected colonies of *H. influenzae* if the growth is seen in the agar plates as the colonies are close to the column of *S. aureus* and the growth is greater than that farther away from it [8].

2.3. DNA Extraction and Molecular Detection of *H. influenzae*

The DNA extraction method was performed according to the manufacturer’s protocols of the genomic DNA purification kit (Promega, USA). The pathogen suspected to be *H. influenzae* isolates was subjected to conventional PCR method to detect the specified gene amplification products of the *omp6* gene for confirming isolation of *H. influenzae*, and the method was done as described previously [9]. The itemized sequences of the primer sets are illustrated in Table 1. A total volume of the reaction mixture (20 µl) contained 5 µl of DNA template, 3 µl of each primer, 5 µl of Green Master Mix (2×), and 4 µl of nuclease-free water. The thermal cycling condition usually starts the process with initial

denaturation steps being carried out at 95°C for 10 minutes. Next, 30 cycles consisted of a denaturation step at 95°C for 30 seconds, annealing step at 55°C for 1 minute, and extension step at 72°C for 2 minutes. The final extension step was at 72°C for 5 minutes. The PCR amplification products were observed on a 1.5% agarose gel stained with ethidium bromide for 45 minutes at 70 Volt and visualized by UV transilluminator.

2.4. Molecular Detection of *bexA* and *bexB* genes in *H. influenzae* Isolates

The PCR technique was used for differentiating *H. influenzae* isolates into typeable and nontypeable using the primer pair with the amplicon size specific to *bexA* and *bexB* genes. The itemized sequences of the primer sets are illustrated in Table 1, as previously described in [10,11] with some modifications.

The thermal cycling condition usually starts the process with the “initial denaturation step being carried out at 95°C for 2 minutes. Next, 30 cycles consisted of a denaturation step at 95°C for 30 seconds, primer annealing step at 54°C for 30 seconds, and extension step at 72°C for 45 seconds. Then final extension step was at 72°C for 5 minutes.” The PCR amplification products were observed on a (1.5%) agarose gel stained with ethidium bromide for 45 minutes at 70 V and visualized by UV transilluminator.

3. RESULTS

Among the 150 eye swabs obtained from patients suffering from conjunctivitis, it was found that only 38 isolates (25.3%) were approved as *H. influenzae*. Among these isolates, only 26 (68.4%) were positive by Satellitism test using discs of X and V factors which are required for growth of *H. influenzae*. To confirm isolation of the *H. influenzae*, these 26 isolates were subjected to further detection using a molecular PCR assay to identify of *omp6* gene, and the results revealed that only 24 (92.3%) out of 26 isolates were positive for *omp6* gene as shown in Figure 1.

The PCR method was used for differentiating all the 24 isolates of *H. influenzae* into both encapsulated (typeable) and nonencapsulated (nontypeable) *H. influenzae* using the primer pair with the amplicon size specific to *bexA* and *bexB* genes. The results found that all 24 isolates (100%) were negative (did not contain *bexA* and *bexB*) genes and are considered as nonencapsulated (nontypeable) isolates. The results of the PCR method for detection of the *bexA* gene are shown in Figure 2.

Table 1: The primer sequence and size of the product.

Genes	Primer sequence	Size of amplicon (bp)	Reference
<i>omp6</i>	Forward: 5'-AACTTTTGGCGGTTACTCTG-3'	351	Ueyama <i>et al.</i> [9]
	Reverse: 5'-CTAACACTGCACGACGGTTT-3'		
<i>bexA</i>	Forward: 5'-CGTTTGTATGATGTTGATCCAGAC-3'	343	Falla <i>et al.</i> [10]
	Reverse: 5'-TGTCCATGTCCTCAAATGATG-3'		
<i>bexB</i>	Forward: 5'-GGTGATTAACGCGTTGCTTATGCG-3'	567	Davis <i>et al.</i> [11]
	Reverse: 5'-TTGTGCCTGTGCTGGAAGGTTATG-3'		

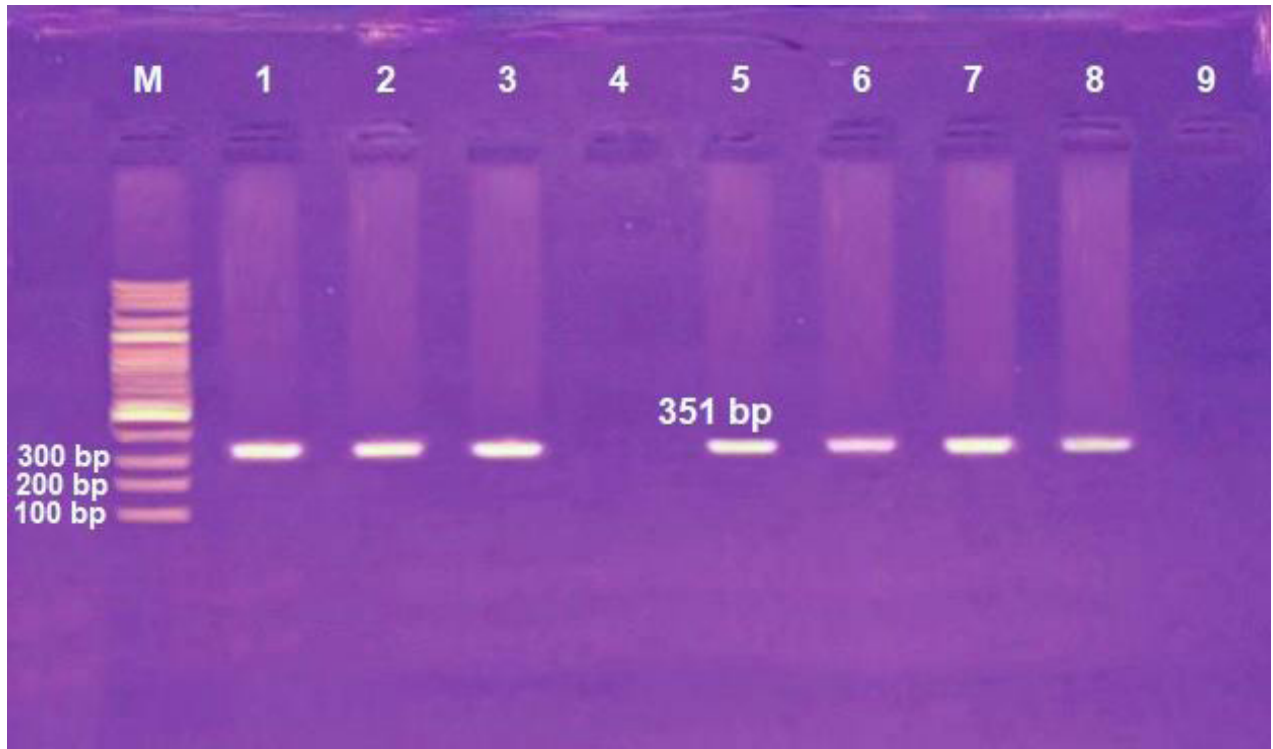


Figure 1: Agarose gel electrophoresis of *H. influenzae omp6* gene detection. Lane M: marker with 100 bp ladder and lanes 1–3 and 5–8: positive for *omp6* gene, while lanes 4 and 9: negative for *omp6* gene. Amplicon size: 351 bp.



Figure 2: Agarose gel electrophoresis of *H. influenzae bexA* gene detection. Lane M: marker with 100 bp ladder and lanes 1–9: negative for *bexA* gene.

Table 2: Profile the three diagnostic methods of *H. influenzae* isolates.

Test type	No. of specimens	No. of positive results (%)
Bacterial culturing	150	38 (25.3%)
Satellitism test	38	26 (68.4%)
PCR technique	26	24 (92.3%)

Also, the comparison and correlation of the three methods used in the diagnosis of *H. influenzae* are summarized in Table 2.

4. DISCUSSION

Haemophilus influenzae is the most common causative organism in children who suffer from acute bacterial conjunctivitis and mostly presents as conjunctivitis-otitis media syndrome coinciding with acute otitis media [12]. The most frequent cause of bacterial conjunctivitis is *H. influenzae*. Complete or partial locus for capsule production is found in some strains of *H. influenzae* but lacks a functional “*bexA*” gene, which encodes for a protein involved in export of capsular polysaccharides to the cell surface. These strains are nontypeable *H. influenzae* (NTHI). Sinusitis, otitis media, conjunctivitis, and chronic obstructive pulmonary disease are mucosal infections, which are mainly caused by nontypeable of *H. influenzae* strains [13].

The findings of the present study showed differences in the rate of *H. influenzae* isolation, probably explained by a variety of factors such as the age of patients, sample size, season of sampling, the type of antibiotic used in treatment, which may be linked to *H. influenzae* expressing multiple virulence factors in numerous locations on the human body as its native host, and the type of detection methods that give sensitivity and specificity in more accuracy. In contrast, the results disagree with the previous results studies reported by Rai *et al.* [14], which showed that the cultural method might be used for *H. influenzae* isolates at a rate of around (18.05%) by using discs of X and V factors, while another study has revealed that the *H. influenzae* can be isolated at a rate of 27.16% by the use of selective media and discs of “X and V” factors [15]. Also, the previous results recorded in Iran found that the *H. influenzae* may be isolated from eye mucus of patients suffering conjunctivitis and other different sites at a prevalence rate (31.4%), depending on their morphology and growth requirement for “X and V” factors [16].

The molecular detection method was used to confirm the diagnosis of the *H. influenzae* isolates by detecting the presence of the *omp6* gene. The *omp6* gene was only detected in 24 isolates (92.3%) of *H. influenzae* obtained from patients with conjunctivitis. Despite the fact that *H. influenzae* has a number of OMPs such as P5 and P2, unlike P2, the P6 protein has a high homology at a rate of 97% in studies of type b and NTHI amino acids, indicating that it is strongly conserved and stable [17]. A previous study had proved that NTHi OMP P6 is a possible part of the vaccine for prevention of *H. influenzae* infections [18].

Additionally, the results revealed that all 24 isolates (100%) were noncapsulated (nontypeable) isolates by using the PCR method. The obtained result is higher than the result of the other research findings obtained from Ref. [19], which indicated that NTHi

isolates account for 44%–68% of bacterial conjunctivitis cases in children. Other studies discovered that the existence or absence of the *bexA* gene determined if the isolate was encapsulated or nonencapsulated by molecular PCR assay; their findings showed that the rate of NTHi was about 93.5% [20].

5. CONCLUSIONS

NTHi was an important bacterium detected in eye swabs of patients suffering from conjunctivitis of both sexes. The use of the PCR method for the identification of *H. influenzae* isolates by detecting the *omp6* gene was regarded as a faster, more sensitive, and more appropriate method than the use of traditional bacterial culturing methods and presumptive identification using X and V factor tests.

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7. AUTHORS' CONTRIBUTION

All authors have made substantial contributions to the conception or design of the work, collection of the study samples, performing all the analysis and laboratory tests, interpretation of data for the work, preparation of the draft, revision of the article, and final approval of the version to be published.

8. CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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10. ETHICAL APPROVALS

This study was approved by the Research Ethical Committee of both Ministry of Health and Environment and Ministry of Higher Education and Scientific Research in Iraq.

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