RESEARCH ARTICLE

Genetic Sequence of Drug-Resistant *Haemophilus influenza* Isolated from Respiratory Tract Infections

Yousor Majid Jameel1*, Rana S. Nasir2

1Department of Optometry, Medical-Technical Institute, Almansour Middle Technical, University, Iraq (MTU)
2Department of Medical Laboratory Techniques, College of Health and Medical, Medical Technical University, Baghdad, Iraq (MTU)

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**ABSTRACT**

This work was done during the period from 1st May 2019 to 15th January 2020. By using the concentration of the inhibitor that was carried out for the *Haemophilus influenza* bacteria, isolates were identified that 50 samples of bacterial colonies of these bacteria, it was found that all isolates were resistant to ampicillin, cefuroxime, pipracilline, cefotoxone, erythromycin, and azithromycin. The counter-current immunoelectrophoresis (CCIE) test and the latex agglutination method have been employed for this purpose. These tests have aided in establishing a rapid diagnosis of *H. influenzae* type b infections in some cases, the positive isolates were identified for those infected with the bacteria were CCIE 7/10, 3/3, 0/5, 0/1, and 0/4, while the co-agglutination was 6/10, 3/3, 0/5, 0/1, and 0/4. The standard phenol/chloroform extraction was used to purify DNA from the sputum’s pellet, followed by precipitation of ethanol. Each reaction set involved sterile water in place of DNA as a negative control. There is a change in the genetic sequence of drug-resistant isolates, CTT to CTA and TTT to TAT.

**Keywords:**Appear respiratory tract, Drug-resistant, *Haemophilus influenza*, Sequence.

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**INTRODUCTION**

Performing the capsular typing of *H. influenza* is very necessary because capsules are the main virulence factors in the disease pathogenesis. The microorganism was categorized depending on the capsular polysaccharide into six serotypes (a–f) in addition to the non-capsulated type.1 *H. influenza* capsular type can be identified by the slide agglutination test (serotyping) through the use of the type-specific anti-sera test.2 Researchers have incorporated the polymerase chain reaction (PCR) for capsular typing in their study because serotyping is not a fully reliable procedure. For the detection of *H. influenza*, gene amplification involved in the capsule expression seemed to be specific, highly sensitive, and rapid.3 Due to the limited information on the burden of *H. influenza* in developing countries, the study was designed for the determination of prevalence, genotype, and antimicrobial susceptibility pattern of *H. influenza* among the respiratory tract infections. This microorganism is known to be carried in the young children’s nasopharynx.4 Encapsulated *H. influenza* type b strains are the main invasive bacterial pathogens, while the non-typeable *H. influenza* (NTHi) is the main type of respiratory infections. The NTHi was found most frequently associated with lung diseases, like the recurrent exacerbation of COPD.5 *H. influenza* type b remains the main concern in developing countries, as the prevalence of invasive infections was much higher than those in the developed countries during the pre-vaccination era.6 Implementing the *H. influenza* type b vaccination led to a reduction of the *H. influenza* type b disease incidence with a relative upsurge in the occurrence of other capsular types (a, c–f) and the non-capsulated strains.7 Until the recent past, drugs of the penicillin group, like ampicillin/amoxicillin were commonly applied in empirical treatments of *Haemophilus* diseases. The 3rd generation of cephalosporins, such as, ceftriaxone has been used after the occurrence of ampicillin resistance in causative microorganisms.8 In different parts of the world, *H. influenza* isolate resistance to β-lactam antibiotics is most often followed by trimethoprim-sulfamethoxazole administration.9

**MATERIALS AND METHODS**

**Culturing**

On agar plates, 50 bacterial cultures of *H. influenza* was performed from sputum samples taken from patients, who attended Baghdad Teaching Hospital during the period from
1st May 2019 to 15th January 2020. Cultures were done on chocolate agar with the addition of X (hemin) and V (nicotinamide adenine dinucleotide) factor in a CO₂ enriched incubator at 37°C. The growth on blood agar was achieved only after appearance of satellite phenomena surrounding other bacteria. *H. influenza* colonies appeared as pale grey, convex, smooth, or transparent. *H. influenza* was shown as gram-negative cocobacilli when microscopically examined after gram staining. The cultured microorganism was further characterized by the oxidase and catalase tests, which should give positive result. To differentiate between capsular polysaccharide *H. influenza* b and non-capsulated species, further serological tests will be of value.

**CCIE Test**
The CCIE test and the latex agglutination method have been employed for this purpose. These tests have aided in establishing a rapid diagnosis of *H. influenza* type b infections in some cases.

**Genotypes**
The *H. influenza* type a (ATCC, 9006), *H. influenza* type c (ATCC, 9007), and non-typeable *H. influenza* (NTHi) (ATCC, 49766) have been used as positive controls for genotype II, positive control for genotype I, and negative control, respectively, in PCR assays. Identification of *H. influenza* species and capsular typing of encapsulated *H. influenza* by PCR. To confirm species identification, PCR amplification was performed to detect the *omp6* gene from genomic DNA. Additionally, the PCR amplification was further carried out using the primer pair specific to *bexA* genes (HI-1 and HI-2) to distinguish encapsulated *H. influenza* strains from NTHi strains. For capsular typing of encapsulated *H. influenza*, six primer sets specific for capsule types a through f was used for all strains, which recognize sequences in capsule-specific genes located within region two of *cap* loci. Isolates containing both the *bexA* gene and one of the cap-specific genes are designated as the specific capsule type. In contrast, isolate that lack both *bexA* and any of the other cap genes are regarded as NTHi, and those which contain cap genes but not *bexA* genes, are regarded as capsule deficient *H. influenza* type b or Hib⁻ strains.

**Capsular Genotyping of Hib Strains**
To determine the capsular phenotypes or phylogenetic relationships of the Hib strains, PCR amplification was performed with two sets of primers. In this regard, two oligonucleotide primer pairs HiHcsA12667F-I and HiHcsA13116R-I, as well as, HiHcsA12668F-II and HiHcsA13484R-II were used to detect capsular genotypes I and II of *H. influenza* type b, respectively. The primers HiHcsA12667F-I and HiHcsA13116R-I permitted the amplification of a 450-bp DNA fragment, whereas another set of primers, HiHcsA12668F-II and HiHcsA13484R-II allowed the amplification of an 817-bp, show in Table 1.

To discriminate between the two capsular genotypes, type I and type II, two separate PCR amplifications were performed for each *H. influenza* type b strain. The PCR reactions were carried out in the final 25 μL volume containing a buffer of 2.5 μL of 10 × PCR, 1.5 μL of 25 mM MgCl₂, 0.2 μL of 5 U/μL Taq DNA polymerase (Genet Bio, Korea), 0.5 μL of each 10 μM primer, 0.5 μL of 10 mM dNTP (Genet Bio Company, Korea), 17.3 μL of double-distilled water, and 2 μL of genomic DNA. PCR conditions were as follows: initial denaturation for 15 minutes at 95°C, 30 cycles of denaturation for 30 seconds at 95°C, annealing for 1-minute at 52°C; elongation for 1-minute at 72°C, and the eventual extension step for 7 minutes at 72°C.

**Gel Electrophoresis of PCR Products**
Six μL of the PCR product was mixed with 2 μL loading buffer and loaded into individual wells of 1% agarose gel, then electrophoresis was done for 45 minutes at 100 V. Further, sizes of the amplicons were measured by comparison with

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Target</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1-(F)</td>
<td>AACTTTTGGGCGGTTACTCTG</td>
<td>Omp6</td>
<td>351</td>
</tr>
<tr>
<td>R1-(R)</td>
<td>CTAACACTGACGACGTTTT</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HI-1 (F)</td>
<td>CGTTTGTATGATGGATCCAGAC</td>
<td>bexA</td>
<td>343</td>
</tr>
<tr>
<td>HI-2 (R)</td>
<td>TGTCCATGTCTTCAAAAAGATG</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HiHcsA12667F-I</td>
<td>GTACCTTGTCACTGCACAAACCTTT</td>
<td>hcsA-I</td>
<td>450</td>
</tr>
<tr>
<td>HiHcsA13116R-I</td>
<td>GGTATATTGAAAAATATGCTGCA</td>
<td>-</td>
<td>-</td>
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<tr>
<td>HiHcsA12668F-II</td>
<td>TGCTTGTCATCGATCAGCAAA</td>
<td>hcsA-II</td>
<td>817</td>
</tr>
<tr>
<td>HiHcsA13484R-II</td>
<td>ACTAAAGAAAGGGGTTGCAA</td>
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<td>-</td>
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</table>

Table 2: Primer sets used in this study

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
<th>Nucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p2</td>
<td>p1-5’</td>
<td>5’-GGCGAGGTTTTATACGACCG-3’</td>
</tr>
<tr>
<td>p2</td>
<td>p1-3’</td>
<td>5’-ATGAAAGAAGGCTTAAAACG-3’</td>
</tr>
<tr>
<td>p6</td>
<td>p6-6’</td>
<td>5’-ATGAAAGAAGGCTTAAAACG-3’</td>
</tr>
<tr>
<td>p6</td>
<td>p6-3’</td>
<td>5’-GGCGAGGTTTTATACGACCG-3’</td>
</tr>
</tbody>
</table>
3 μL of the 100-bp DNA ladder. The resulting PCR products were stained with ethidium bromide for 20 minutes and visualized under UV illuminations. To confirm whether amplified bands corresponded to \( hcsA \) type I or \( hcsA \) type II fragments, one of the PCR products from each gene fragment were randomly sequenced (TAG Copenhagen, Copenhagen, Denmark) (Table 2).

Using the platinum taq DNA polymerase (Invitrogen, Carlsbad, CA), a single colony of \( H.\text{influenzae} \) was alternatively suspended in 100 μL of sterile water, then 1 μL of the suspension was used as a template in PCR. After a 3-minute initial incubation at 94°C, reactions composed of 30 cycles at 94°C for 30 seconds, 55°C for 1-minute and 72°C for 30 seconds. The last cycle was then followed by incubation for 3 minutes at 72°C. The PCR purification kit (Qiagen, Valencia, CA) was used for amplicon purification.

**RESULTS**

**Drugs Resistance \( H.\text{influenzae} \)**

After making the lowest concentration of inhibition on influenza isolates, it was found that all isolates were resistant to ampicillin, cefuroxime, pipracilline, cefotoxone, erethromycin, and azithromycin, (Table 3).

**DISCUSSION**

\( H.\text{influenzae} \) is one of the dangerous bacteria that infects the lung and causes lung lobes problems that may lead to death, \( H.\text{influenzae} \) isolates, which are resistant to several types of medication, were more virulent for people with this bacterium. The resistance was shown by these bacteria against the different types of effective antibiotics, and this means that the bacteria changed from their characteristics sensitive to drugs. There are several types of resistance to these bacteria.

A genetic change or a plasmid may occur of \( H.\text{influenzae} \), all of which lead to changing the inherited characteristics of those bacteria to resist drugs. This leads to the ability of bacteria to manufacture protein, for example, or to multiply the DNA without the effect of the drug. These findings agreed with Chang HH et al. (2015), who reported the multidrug resistant (MDR) infection is usually significantly harder and more expensive to be treated, and it represents a public health threat. However, for various pathogenic bacteria, various underlying mechanisms are traditionally utilized to clarify those findings, and it is unclear whether each bacterial taxon has its own mechanism for being a multidrug-resistant or whether common mechanisms are found between the distantly-related pathogenic microorganisms. In this study,
we provide a systematic overview of causes for excess MDR infections and define testable anticipations suggested by each hypothetical mechanism, e.g., epidemiological, experimental, population genomic, and other tests of such hypothesis. Genetic sequencing changes gave bacteria the resistance to common and effective treatments with the same degree for many sensitive bacteria, the change between adenine and thymine gave bacteria a resistance package for these drugs, CTT to CTA and TTT to TAT. Köser CU et al. (2014) found that the regimens which have agents to which a single mutation confers cross-resistance must be avoided if those mutations frequently emerge in vivo. WGS has recently reported that this can be the case with the three phase II trial regimen, which has bed clofazimine and aquiline since the cross-resistance to both drugs is conferred to the mutational upregulation of the efflux pump.

REFERENCES