New Satellitism Test and molecular detection of haemophilus influenzae in Respiratory tract infections in mosul, Iraq

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Abstract
Background: *Haemophilus influenza* fastidious microorganisms can not grow on blood agar but can be seen around beta-hemolyt ic colonies of *S.aureus* .In this study, we tested and compared with *S.aureus* and *S.pyogenes* to determine an alternative bacteria to be used for satellite test to identify *H.influenzae* .We collected 120 swabs from different respiratory tract infections the results show that 12(10%) of 102 positive culture was *H.influenzae* and 8(66.6%) were positive by satellite test with *S.aureus* and 11(91.6%) were positive by satellite test with *Streptococcus pyogenes* and 12(100%) approved by PCR assay as *Haemophilus inflenzae*. Based on our findings, the.pyogenes satellitism test is more reliable than the S.aureus test and produces fewer false-negative results.
Keywords: *Haemophilus inflenzae*, Satellitism Test.

1. Introduction

Haemophilus influenzae is gram negative bacteria coccobacilli facultatively anaerobic, needs both hemin (X factor) and nicotinamide adenine dinucleotide (NAD) (V factor) to thrive. [1].*H.influenzae* strains divided into two groups unencapsulated strains (nontypeable haemophilus influenza)NTHi and strains that were encapsulated and were divided into six serotypes (a - f) [2]. It is a part of the indigenous microbiota of the mucous membrane of the upper respiratory tract. However, this bacteria can be an opportunitc pathogen capable of causing infections in respiratory tract ranging from noninvasive acute otitis media to invasive infections such as epiglottis and pneumonia [3] . More than 95 % of the invasive infections are associated with type b encapsulated *H. inflenzae* ,NTHi also play an important role in chronic bronchitis and pneumonia in adults [4]. Satellitism tests are among the most common important methods to confirm H.influenzae [5]. Traditional Satellitism Test with *S.aureus* is not accurate enough to identify *H.influenzae* due to the false-negative results [6] .This study aimed to isolate and identify *Haemophilus influenzae* from respiratory tract infections using a new Satellitism test with Streptococcus pyogenes and PCR technique [7].

2. Material and Methods

Respiratory tract samples

The respiratory tract swabs were obtained from mosul hospitals for the period between first of July and October of 2022. A total of 120 samples were taken and included (sputum , Tonsils swabs, nasal swabs, otitis media swabs, nasopharyngeal swabs) for both sexes between (5-80) yaers . Swabs were taken from the specimens and grown on Blood agar, Gonococcal GC media, MacConky agar, Levienthal's medium, and chocolate agar before being incubated at 37 degrees Celsius for 18 to One whole day in the air containing 5-percent CO2. After that, the nutritional needs of Haemophilus inflenzae to both X,V factors were investigated, and each bacterial isolate was examined using a Gram stain, an oxidase test, and the API-NH system (Biomereiux France).

Satellitism Test

We inoculated blood agar medium and a streak pure culture of S.aureus at 37C°in 5%CO2 for 24 hours after mixing a loopful of suspected bacterial coloies with around 2 ml of sterile saline.. [8].In this study we used the same procedure but a suspension of suspected bacterial coloies inoculated on blood agar with a streak of pure culture of *S.pyogenes*. after incubation, Figures 1, 2, and 3 show how the spread of H. influenzae is limited to the spaces between colonies of S. aureus and S. pyogenes.

figure 1 satellitism with S.aureus

figure 2 satellitism with S.pyogenes

figure 3 false-negative satellitism with S.aureus

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DNA extraction of haemophilus influenzae

Genomic DNA from the studied haemophilus influenzae strains used a technique that included extraction commercially available Molecular biology laboratory equipment for DNA extraction (Geneaid) Procedures for DNA extraction were carried out as per the manufacturer's instructions. [9].

Molecular detection of haemophilus influenzae

The standard PCR used to identify H.influenzae consisted of a 20 ul reaction including 10 ul of master mix, 2 ul of forward and reverse primer, 2 ul of DNA template, and a variable amount of nuclease free water to complete the final volume as shown in table 1. The omp6 gene was utilized to confirm the isolation of H. influenzae, and the BexA gene was used to distinguish between encapsulated and unencapsulated (NTHi) H. influenzae isolates [10]. In addition, the serotypes (a-d) and nontypeable strains (table 2) were confirmed using the Hin-in gene.

Table 1. Components of polymerase chain reaction

No.	Components in a reagent	Volume
1	TruGreen Master Mix	(10µl)
2	Nuclease: free water	(4µl)
3	Primers forward	(2µl)
4	Primers reverse	(2µl)
5	DNA template	(2µl)
	Total volume	(20µl)

Table 2. Primers sequences and thermal cycler conditions

Gene	Primer sequence	Size bp	PCR condition
P6F P6R	AACTTTTGGCGGTTACTCTG CTAACACTGCACGACGGTTT	351	95 C 10 min 1X 95 C 30 sec 55 C 1 min 30X 72 C 2 min 72 oC 5 min 1X 95 C 10 min 1X (31)
Bex A F Bex A R	CGTTTGTATGATGTTGATCCAGAC TGTCCATGTCTTCAAAATGATG	343	95 C 2 min 1X 95 C 30 sec 54 C 30 sec 30X 72 C 45 sec 72 C 5 min 1X
Hi-in-F Hi-in-R	AAAGTGCGGGACTGAGA CCGGTGCTTCTTCTGTAT	313	95 C 2 min 1X 95 C 30 sec 52 C 30 sec 30X 72 C 45 sec 72 C 5 min 1X

3. Results

Among 120 swabs from respiratory tract infections 102 were positive culture. It was revealed that only 12 isolates (10%) were authorized H.influenzae only 8(66.6%) were positive by satellitism test with S. aureus the remaining 4 isolates were false-negative findings where as the satellitism test with S.pyogenes yielded 11(91.6%) positive results for H.influenzae . Twelve potential H.influenzae isolates underwent further testing, this time using a conventional PCR approach designed to identify the omp6 gene. Only three of the isolates tested positive for the BexA gene, while all 12 tested positive for the omp6 gene.

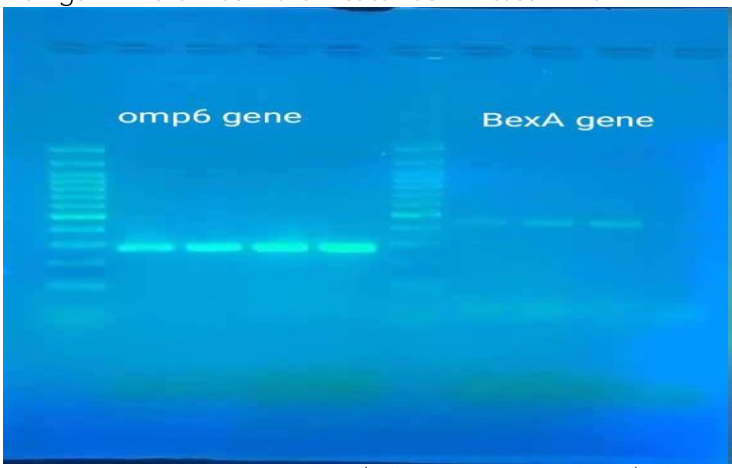

Figure 4 omp6 gene (351bp)& Bex A gene(343 bp)

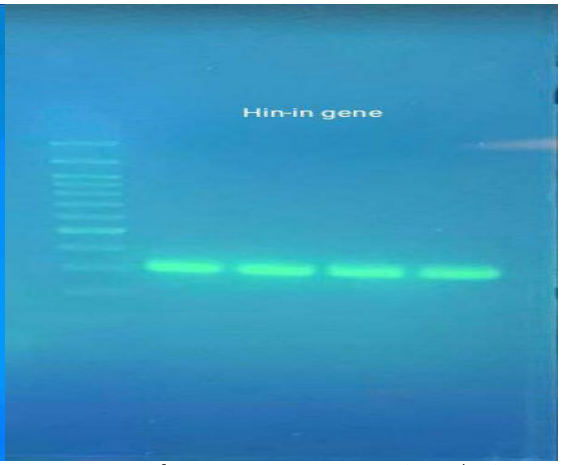

figure 5 Hin-in gene (313 bp)

4. Discussion

H.influenzae is a part of the normal upper respiratory tract flora . However it is the most common pathogen especially in children which

causes many systematic infections It is also major cause of various disease [12]. After introduction of *Haemophilus influenzae* serotype b Hib conjugate vaccine many invasive disease was decreased so the epidemiology of H.influenzae was changed now, the increase of *H.influenzae* diseases caused by (NTHi) [13]. Despite the development of laboratories in the world satellitism test is still the most important test to identify *H.influenzae* because it is fastidious bacterium and any trace amounts of X factor or V factor or heavy inoculum will responsible for the false-negative results [14] . Although the detection of *H.influenzae* by traditional culture methods is cheap and easy but may give wrong results, so using molecular PCR assay to identify *H.influenzae* by selection target, the gene opm6, which encoded the outer membrane protein p6 has highly conserved nucleotide sequence also *BexA* gene which encoded capsulation- associated protein. According to Our research shows that several species of beta hemolytic bacteria besides S. aureus may yield positive findings when tested with suspected Haemophilus colonies, like S. pyogenes, leading to far more accurate results and fewer false negatives in the Satellitism Test.

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