

Detection of β -lactamase in *Haemophilus influenzae* Isolates by Double Disk Synergy Test

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Abstract

A total of 90 isolates of *Haemophilus influenzae* were included in this study. Among them 47.8% were resistant to ampicillin and amoxicillin. Of the 90 isolates 43 were positive for β -lactamase by cefinase test and the rest (47) were β -lactamase negative. Interestingly, all the β -lactamase positive isolates were ampicillin resistant. No isolate of *H. influenzae* was β -lactamase negative ampicillin resistant (BLNAR). All β -lactamase positive ampicillin resistant (BLPAR) isolates were also positive for β -lactamase by double disk (Ampicillin and Amoxicillin/clavulanate) technique. And all β -lactamase negative isolates were also negative double disk synergy test. Both the sensitivity and specificity of double disk synergy test were 100% compared to cefinase test. So, double disk synergy test can be used to detect β -lactamase as an alternative of cefinase test by incorporating an augmentin (amoxicillin/clavulanic acid) disk during antimicrobial susceptibility testing by disk diffusion method.

Keywords: β -lactamase; BLNAR; BLPAR; *H. influenzae*; Double disk synergy test

Introduction

Haemophilus influenzae is a major causative agent of community acquired respiratory tract infections (CARTIs), such as acute otitis media, bacterial rhinosinusitis, chronic bronchitis, pneumonia and meningitis. Approximately 0–30% of *H. influenzae* isolates (average 15%) are β -lactamase-positive [1,2]. Ampicillin/amoxycillin was the most effective treatment option for *Haemophilus* infection until the recent past. Resistance to ampicillin results from the production of a β -lactamase and alteration of the antibiotic target, penicillin-binding protein [3,4]. β -lactamase an enzyme which cleavage the β -lactam ring of ampicillin/amoxycillin antibiotic and make them ineffective in killing bacterial pathogen that produce β -lactamase. As a result the bacterial pathogens showed resistance to β -lactam antibiotic, such as ampicillin, amoxicillin, benzyle penicillin etc. Many bacterial pathogens such as *H. influenzae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, etc. have been reported to produce β -lactamase. Treatment problems linked to the prevalence of β -lactam resistance are compounded by the frequency of combine resistance to many other antibiotics in addition to penicillin, such as chloramphenicol, cotrimoxazole, tetracycline etc [5]. Recent studies indicate the increasing trend of ampicillin resistance among *H. influenzae* isolates from the different part of the world [6-8]. For better management of invasive infections due to β -lactamase mediated ampicillin (penicillin) resistant bacterial pathogens, it is important to detect β -lactamase enzyme accurately as well as cost effectively. We standardized a method for β -lactamase detections simultaneously during the testing of antimicrobial susceptibility by incorporating an additional disk augmentin (amoxicillin/clavulanic acid). The double disk synergy method is very effective in detection of β -lactamase producer and eliminated the additional cost for β -lactamase detection by cefinase disk test.

Materials and Methods

Bacterial strains

A total of 90 isolates of *H. influenzae* were included in this study. Of the 90 isolates 43 were ampicillin resistant and the rest were susceptible to ampicillin by disk diffusion and E-test technique.

Antimicrobial susceptibility

Antibiotic susceptibility was qualitatively determined by disk diffusion method according to Standard Clinical laboratory Institute (Previously National Committee for Clinical Laboratory Standard; NCCLS) guideline [9]. Minimum inhibitory concentration (MIC) was also determined E-test according to Jorgensen et al., [10,11] technique using antimicrobial strips of ampicillin, amoxicillin, azithromycin, trimethoprim-sulfamethoxazole (TMP-SMX), ciprofloxacin, chloramphenicol, ceftriaxone, (AB Biodisk, Solna, Sweden). *Haemophilus* testing media (HTM) supplemented with X and V factor was used for susceptibility testing of *H. influenzae*. The plates were incubated in candle extinction jar (10-15% CO₂) at 37°C for 24 h prior to reading of the results. MICs were interpreted by using Standard Clinical laboratory Institute breakpoints. The MIC was defined as the lowest concentration of antibiotic that inhibited the visible growth. Isolates were considered multi-resistant if they had reduced susceptibility to at least three structurally unrelated antibiotics. *H. influenzae* ATCC 49247 strains was used for the quality control.

β -Lactamase Detection Assay

Double disk synergy test (DDST)

This test was performed on *Haemophilus* test medium (HTM) by using ampicillin and augmentin disk in addition to other antibiotic disks in the same antimicrobial susceptibility testing plate. The antibiotic disks containing bacterial culture plates were incubated at 37°C for 20-22 hours. The plates were interpreted after incubation in three different

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Received October 29, 2015; Accepted November 16, 2015; Published November 23, 2015

Citation: Rashid H, Rahman M (2015) Detection of β -lactamase in *Haemophilus influenzae* Isolates by Double Disk Synergy Test. J Microb Biochem Technol 7: 417-418. doi:10.4172/1948-5948.1000248

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ways. If the test isolates were susceptible to ampicillin and augmentin, no possibility of β -lactamase production, secondly if the isolate found to be resistant to ampicillin and susceptible to augmentin interpreted as β -lactamase positive ampicillin resistant (BLPAR) and finally if the test isolates showed resistance to both ampicillin and augmentin, were regarded as β -lactamase negative ampicillin resistant (BLNAR).

Cefinase test

β -lactamase production in *H. influenzae* was determined using cefinase stick (nitrocefin) assay (Cefinase) (11). Quality control testing was conducted daily according to the manufacturer's instructions. Results were reported only when quality control results had been accepted.

Results

A total of 90 isolates (20 from CSF, 15 from blood and 55 from Nasopharyngeal swab) of *Haemophilus influenzae* were included in this study. Among the ninety isolates 47.8% were resistant to ampicillin or amoxicillin. Minimum inhibitory concentration of ampicillin resistant isolates was 4->256 $\mu\text{g/ml}$ for ampicillin and 8->256 for amoxicillin (Table 1). Of the 90 isolates 43 (47.8%) were positive for β -lactamase by cefinase test and the rest of 47 isolates were β -lactamase negative. All the β -lactamase positive isolates were ampicillin resistant. No *H. influenzae* isolate was β -lactamase negative ampicillin resistant (BLNAR). All β -lactamase positive ampicillin resistant (BLPAR) isolates were also positive by DDST. Consistently all β -lactamase negative isolates were also negative for β -lactamase by DDST. Both the sensitivity and specificity of DDST are 100% compared to cefinase test (Table 2).

Discussion

H. influenzae especially serotype b is one of the major cause of severe invasive infections, i. e., pneumonia, meningitis and septicemia among the extreme age group (<5 years and >65 years). Ampicillin/Amoxicillin were the most effective treatment option for *Haemophilus influenzae* infection until the recent past. Development of ampicillin resistance made the treatment difficult and led the physician to think about other options. Resistance to ampicillin results from the production of a β -lactamase and alteration of the penicillin-binding protein. Treatment problem linked to the prevalence of β -lactamase mediated resistance are compounded by the frequency of cross-resistance to many other antibiotics. So, it is very important to know the mechanism of ampicillin/amoxicillin resistance and the prevalence of β -lactamase mediated ampicillin resistance among the clinical isolates of *H. influenzae* circulating in our community to develop an empirical treatment strategy.

Antibiotics	MIC range ($\mu\text{g ml}^{-1}$)		Break point value ($\mu\text{g ml}^{-1}$)		
	Susceptible	Resistant	S	I	R
Ampicillin	0.12- \leq 1	\geq 4->256	\leq 1	2	\geq 4
Amoxicillin	0.25- \leq 2	\geq 8->256	\leq 2	4	\geq 8

Table 1: Susceptibility and MIC result of colonized isolates of *H. influenzae*.

Cefinase stick test	Double disk synergy test		Total
	Positive (n=43)	Negative (n=47)	
Positive	43	0	43
Negative	0	47	47
Total	43	47	90

Specificity 100% and sensitivity 100%

Table 2: Comparison of two beta-lactamase testing methods for *H. influenzae* isolates.

Nitrocefin disk test was generally used for the detection of β -lactamase, which produce pink color by β -lactamase positive isolates and remain unchanged if the strain is β -lactamase negative. Sometime β -lactamase positive isolates produce color not strong enough to detect visually and very confusing to conclude the result. But in case of double disk method the results could be obtained by the presence clear zone of inhibition without any chance to be confused. Moreover, this test could be accompanied with antimicrobial susceptibility testing without additional cost and efforts. This method allows detecting the ampicillin susceptibility as well as the mode ampicillin resistance simultaneously. So, the DDST can time, labor and additional cost for nitrocefin disk test. Sensitivity and specificity of double disk method is 100% compared to nitrocefin disk test. So, DDST can be used for the detection of β -lactamase effectively fin *H. influenzae* as well as other β -lactamase producing bacteria such as *Moraxella catarrhalis*, *Staphylococcus aureus*, etc.

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