β-LACTAMASE ENZYME ACTIVITIES OF HAEMOPHILUS INFLUENZAE ISOLATES WITH ELEVATED MICs TO AMOXICILLIN AND AMOXICILLIN / CLAVULANIC ACID

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SUMMARY

Haemophilus influenzae is recognized as a frequent cause of a variety of infections among outpatients. Antimicrobial resistance of Haemophilus influenzae to ampicillin and amoxicillin is usually due to the β-lactamase production. This organism produces either of two β-lactamases, TEM-1 or ROB-1 that both have different pI points. Very rarely a non-β-lactamase mechanism of resistance, attributed to altered penicillin binding proteins (PBPs) does occur. The strains exhibiting this form of resistance are referred to as BLNAR (β-lactamase-negative, ampicillin resistant) strains, and are also resistant to amoxcillin/clavulanic acid and some other β-lactams. Recently, BLPACR (β-lactamase-positive, amoxicilline-clavulanate resistant) strains have been described. This study was conducted to investigate the possible resistance mechanisms of these strains. The haemophilus influenzae strains were divided into two groups as Group A; BLPACS (β-lactamase positive, amoxicilline-clavulanate susceptible, n=8) and Group B; BLPACR (n=15). In order to investigate the resistance mechanism which could be due to the increased enzyme activities, β-lactamase enzyme activities were determined using nitrocefin as substrate. We also examined their pI points using isoelectiric focusing to distinguish different types of these enzymes. β-lactamase enzyme activities were not different in both groups (Specific activity; Group A; x :0.97 ± 0.32 μmol/min/mg prot., Group B; x : 1.17 ± 0.45 μmol/min/mg prot.). Their isoelectiric focusing patterns were also identical with the pI points 5.1. Our findings showed that increased enzyme activity was not the mechanism for resistance in BLPACR strains. The resistance mechanism in these strains remains to be elucidated.

Key words: *Haemophilus influenzae, β-lactamase, amoxicilline-clavulanate*

ÖZET

Hemofilus influenza, hastaneye başvuran hastalarda infeksiyonların sık görülen bir nedeni olarak tanınır. Hemofilus influenzanın ampisilin ve amoksisiline karşı antimikrobiyal direnci, genellikle bu bakterinin β-laktamaz üretimine bağlıdır. Bu organizma farklı pI noktalarına sahip iki β-laktamaz enziminden ya TEM-1 veya ROB-1 enzimini üretir. Nadir olarak non-β-laktamaz direnç mekanizması, değişmiş penisilin bağlayıcı proteinlere atfedilir. Bu tür direnç gösteren suşlar, BLNAR (β-lactamase-negative, ampicillin resistant) olarak adlandırılır. Yakın zamanlarda amoksisilin-klavulanik aside dirençli suşlar tanımlandı (BLPACR, β-lactamase-positive, amoxicilline-clavulanate resistant). Bu çalışma, bu suşlardaki olası direnç mekanizmasını araştırmak için yapıldı. Hemofilus influenza suşları Grup A; BLPACS (β-lactamase positive, amoxicilline-clavulanate susceptible, n=8) ve Grup B; BLPACR (n=15) olarak iki gruba ayrıldı. Direnç mekanizmalarından biri olabilecek enzim aktivitelerindeki artışı saptamak için nitrosefin ile β-laktamaz enzim aktiviteleri tayini yapıldı. Ayrıca, izoelektirik fokuslama ile bu enzimlerin farklı türlerinin olup olmadığı araştırıldı. β-laktamaz enzim aktiviteleri, iki grup arasında farklı olmadığı belirlendi (Spesifik aktivite; Grup A; x :0.97 ± 0.32 μmol/min/mg prot., Grup B; x : 1.17 ± 0.45 μmol/min/mg prot.). Ayrıca, izoelektirik fokuslama karakteristikleri de benzer olarak saptandı (pI; 5.1). Bu sonuçlar, enzim aktivitelerindeki herhangi bir artışın, BLPACR suşlarında ortaya çıkan direncin mekanizması olmayacağını göstermektedir.

Anahtar sözcükler: *Hemofilus influenza, β-laktamaz, amoksisilin-klavulanik asid*

Haemophilus influenzae is an aerobic pleomorphic gram-negative coccobacillus and has become increasingly resistant to beta-lactam antibiotics (1,2). Three major mechanisms, both enzymatic and non-enzymatic, are involved. Enzymatic resistance is mainly due to the production of a TEM-1 plasmid-mediated beta-lactamase, and in some cases to a new enzyme ROB-1. Of the non-enzymatic mechanisms, decreased permeability due to alteration of outer membrane proteins seems to be rare in comparison to decreased affinity of penicillin-binding proteins for beta-lactam antibiotics. Enzymatic resistance is present in about 10-20% of clinical isolates, while non-enzymatic resistance is present only in 2-4% (3).

During the last 10 years, a continuous increase in the incidence of beta-lactamase-producing Haemophilus influenzae has been observed (4). The production of beta-lactamase is the most important mechanism of bacterial resistance to beta-lactam antibiotics. Virtually all bacteria have the capability of synthesizing the enzyme. Microorganisms may already possess the native genetic information necessary for beta-lactamase production (i.e., chromosomal), or may acquire the capacity by transfer of DNA from another organism (i.e., plasmid-mediated). The level of beta-lactamase production may be stable and noninducible (constitutive enzyme production), or may be stimulated on exposure to selected beta-lactam antibiotics (inducible enzyme production). Many Hemophilus influenzae possess beta-lactamases that hydrolyze penicillins and cephalosporins. The most common plasmid-mediated beta-lactamase is the TEM enzyme, which is present in Hemophilus. These enzymes hydrolyze the amide bond in the beta-lactam ring of the compound, producing acidic derivatives that have no antibacterial properties (5). One technique to overcome bacterial resistance has been the development of beta-lactamase inhibitors. Clavulanic acid is a beta-lactamase inhibitor that inhibits the beta-lactamases of Hemophilus. Clavulanate acts as a "suicide" inhibitor, forming a stable enzyme complex that binds to serine at the active site of the enzyme. Clavulanate readily crosses the outer cell wall of most Enterobacteriaceae to interact with beta-lactamases in the periplasmic space (6-8).

Resistance of bacteria to beta-lactam antibiotics has become a serious problem in the past several decades (7). In order to investigate the resistance mechanism of BLPACR strains that might have higher enzyme activities, we aimed to determine β-lactamase enzyme activities. We also examined their pI points using isoelectiric focusing to distinguish different types of these enzymes.

MATERIALS and METHODS

Twenty-three untypeable H. Influenzae isolates that were obtained from various specimen sources from outpatients were included in this study. Eight of them were β-lactamase-positive, amoxicilline-clavulanate susceptible (Group A) and fifteen of these isolates were β-lactamase-positive, amoxicilline-clavulanate resistant (Group B). All strains were stored at -70°C and subcultered at least

twice before testing (9). These strains were tested in triplicate by Etest. Their Etest MICs (minimum inhibitory concentrations) were =8/4 μg/ml for resistant strains, =4/2 μg/ml for susceptible strains. HTM (Haemophilus Test Medium, Becton-Dickinson Cockeysville, MD, USA) was used for growth and determination of MIC. Plate cultures were incubated at 37°C in 5% CO_2, whereas liquid cultures were incubated at 37°C in room air and shaken at 200 cycles / min. H. Influenzae strains were grown in 20 ml HTM broth for 16 - 18 hours. They were centrifuged (Beckman JA20) at 10000 g for 10 minutes to isolate cells. Bacterial pellet was suspended in 1 ml 10 mM phosphate buffer (pH; 7.0). For preparation of cell free extract, the cells were ruptured by ultrasonic treatment in a Branson sonifier at 4°C. Cell debris was removed by centrifugation. The supernatant was used for all β-lactamase enzyme tests and isoelectiric focusing. β-lactamase activities were determined using nitrocefin as substrate by the spectrophotometric method (10). The decrease in absorbance of the substrate at 550 nm was measured in a temperature controlled spectrophotometer (Beckman Acta III). Specific β-lactamase enzyme activity was defined as the amount of the enzyme which formed μM nitrocefoic acid per minute per protein. Protein determination was made by the method of Lowry (11). The pIs of the enzyme were determined by isoelectiric focusing in polyacrylamide gels, pH; 3.5 to 10 ampholine (Isolab resolve IEF gels, Isolab inc, Akron, OH, USA). Samples (up to 20 μl, 10 mg/ml) were applied near the anode. Isoelectiric focusing was carried out at 400 to 1500 V for 1 hr. Temperature was maintained between 5-10 °C during the procedure. β-lactamase bands were identified by flooding gels with nitrocefin (500 mg/ml). To evaluate their pI values, a Bio-Rad standard was used (pI; 4.3). All extracts were tested three times. Data was analysed statistically using Student's t test (mean ± SD) by SPSS statistical analysis software in a personal computer. This study was conducted in the research laboratories of Veteran Hospital, Cleveland, USA.

RESULTS

The specific enzyme activities of twenty-three H. Influenzae isolates that eight of them were β-lactamase - positive amoxicilline - clavulanate susceptible (Group A) and fifteen of them were β-lactamase - positive amoxicilline - clavulanate resistant (Group B) were determined. The mean specific activity of β-lactamase enzyme in Group A was 0.97 ± 0.32 μmol/min/mg prot., and of Group B was 1.17 ± 0.45 μmol/min/mg prot. (Figure 1). Results of isoelectiric focusing experiments are represented in Fig. 2. As can be seen, all pIs were in the acid range. pI values of the enzymes in Group A and B were identical (pI; 5.1) in isoelectiric focusing.

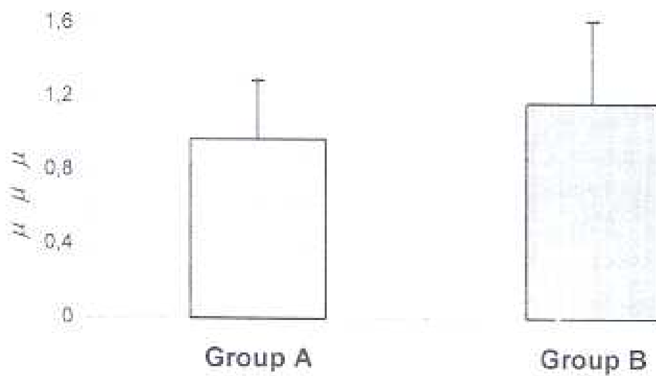

Figure 1. Specific β-lactamase enzyme activities (μmol/min/mg prot.) in Group A and B (mean ± SD, p>0.05)

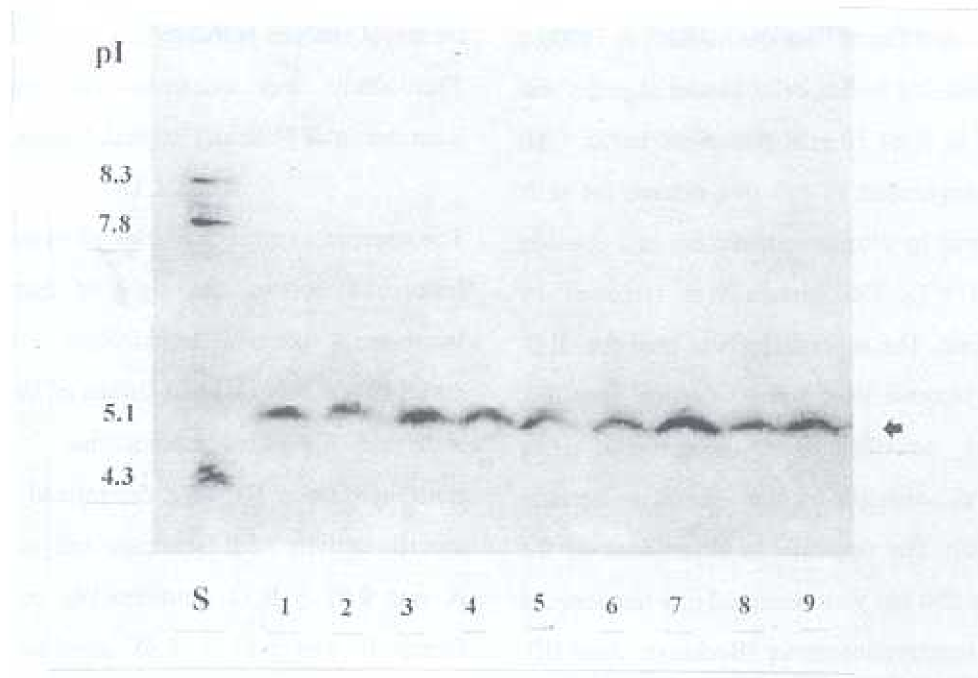

Figure 2. Isoelectiric focusing of enzyme extracts of Group A (BLPACS; 1,2,3,4,5) and Group B (BLPACR; 6,7,8,9).

DISCUSSION

The resistance of bacteria to beta-lactam antibiotics is usually associated with the production of the enzyme, beta-lactamase, which inactivates the beta-lactam molecule. Beta-lactamases constitute the major defense mechanism of pathogenic bacteria against beta-lactam antibiotics. When the beta-lactam ring of this antibiotic class is hydrolyzed, antimicrobial activity is destroyed (12). Although beta-lactamases have been identified with clinical failures for over 40 years, enzymes with various abilities to hydrolyze

specific penicillins or cephalosporins appear more frequently in clinical isolates. In the long search for inhibitors of bacterial beta-lactamase the first clinically useful agent, clavulanic acid, was isolated as a metabolite of Streptomyces clavuligerus. Clavulanic acid prevents antibiotic degradation by the beta-lactamases of many clinically significant pathogens. This molecule eventually inactivates the target enzymes permanently. A combination of clavulanic acid with amoxicillin demonstrates clinically significant activity against many bacteria, including H. influenzae, S. aureus, B. fragilis, streptococci, and is useful in the treatment of upper respiratory, urinary tract, and skin and soft tissue infections (5,13).

Amoxicillin / Clavunate resistant β-lactamase producing strains of H. Influenzae seem to be rare, although β-lactamase positive strains were recently reported (9,14,15). In this study, eight β-lactamase - positive, amoxicilline - clavulanate susceptible strains with MICs =4/2 μg/ml and fifteen β-lactamase - positive, amoxicilline -clavulanate resistant strains with MICs =8/4 μg/ml were analysed. The mechanism of the resistance to amoxicillin / clavunate was investigated in these strains. The β-lactamases produced by Gram-negative bacteria are known to differ in their substrate specificity, inhibitor profiles, electrophoretic mobilities, biochemical properties. Potential explanations for the mechanism of resistance include hyperproduction of TEM-1 or ROB-1 β-lactamase, production of an altered TEM-1 or ROB-1 β-lactamase that has become resistant to clavulanate inhibition, production of a completely novel β-lactamase that is not effectively inhibited by clavulanate, or elaboration of altered penicillin binding proteins (13,16,17). To evaluate the enzyme activities in Group B that these strains could have higher β-lactamase enzyme activities so that β-lactamase would not be inactivated by clavunate completely, the β-lactamase enzyme activities were measured in β-lactamase positive amoxicilline - clavulanate susceptible and β-lactamase-positive, amoxicilline-clavulanate resistant H. influenzae isolates using nitrocefin as substrate. It was found that the enzyme activities were not different statistically. We also examined the pI values of these enzymes by isoelectiric focusing to assess their electrophoretic mobilities of enzymes in both groups. This technique of separation and specific staining of β-lactamases allows demonstration of low levels of activity and presents the different enzymes produced by various strains as patterns of bands that can easily be recognised and compared. Isoelectiric focusing patterns of cell extracts in both groups were identical with pI points 5.1 for β-lactamases.

Other enzymatic antibiotic modifications and non-enzymatic mechanisms have not yet been elucidated in H. influenzae with BLPACR. These may include a permeability barrier or altered penicillin binding proteins (PBSs) (18). It was concluded that the mechanism of resistance was not related to the enzyme activities and also to the

different enzyme types. It remains to elucidate the mechanism of resistance in β-lactamase-positive, amoxicilline-clavulanate resistant H. influenzae strains.

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