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DRIFTED CATALYTIC PROPERTIES OF β-LACTAMASES DUE TO UNCONSTRAINED USE OF ANTIBIOTICS

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Abstract

Context: Antibiotic resistance is an old problem with new face as the rate of infections due to multidrug resistant bacteria is increasing everyday and the number of new antibiotics to overwhelm the problem is becoming smaller. Major mechanism beneath this growing resistance is concomitant with the changes in β-lactamases catalytic activity and its functional enhancement.

Objectives: In β-lactamases secreting clinical isolates at least 10% are extended-spectrum β-lactamases (ESBL) that are not even treatable with β-lactamases inhibitor like clavulanic acids. This implies that the catalytic domains of β-lactamases have been mutated towards higher pathogenicity. The aim of the present study is to define the changes in β-lactamases catalytic efficiency against β-lactam antibiotics and its inhibitors.

Materials and Methods: In this research work we have used multiple drug resistant (MDR) strains from surgical site of infections. A rapid method was used for specific detection of bacterial β-lactamases that uses β-lactam antibiotics as substrates. In this, the end products (open beta-lactam ring forms) generated after separately incubating substrates with β-lactamases producing strains. Those end products of antibiotics were highly fluorescent after specific treatment and could be analyzed visually under long-wave UV lamp for efficiency.

Results: β-lactamases secreting strains are variably capable of defending β-lactam antibiotics. Interestingly, one of the *E. coli* strain secretes ESBL, this means that the strain is resistant against clavulanic acid. However, the most fascinating fact of the finding is that ideally the β-lactamases supposed to hydrolyze Penicillin by default but in our isolates, β-lactamases are not able to hydrolyze penicillin instead they hydrolyze amoxicillin, a derivative which replaced clinical use of penicillin. In addition to that we have identified the presence of New Delhi Metalo- beta-lactamase in one of the clinical isolates.

Conclusion: Rate of evolution in microbes is very high. Thus we presume that some of the amino acids in the functional domain of β-lactamases have been changed respective to extinct use of penicillin whereas it is effective against clinically used other beta lactam antibiotics.

Keywords: β –lactamase, β –lactam antibiotics, MDR strains, NDM 1.

Introduction

The accumulative increase of drug resistance among both Gram-positive and Gram-negative bacteria represents a mounting encounter for the development of new antimicrobials. The pace of antibiotic drug development has been decelerated since the last decade and, especially for Gram-negatives, clinicians have been facing a dramatic shortage in the availability of therapeutic options to face the emergency of the resistance problem throughout the world. In this alarming scenario, although there is a shortage of new antibacterial molecule reaching the market in the near future, antibiotic discovery remains one of the key issues to successfully stem and maybe overcome the tide of resistance (Bassetti *et al.* 2011). Therefore, infection control policies and optimization in the use of already existing molecules are still the most effective approaches to reduce the spread of resistance and preserve the activity of antimicrobials. However, the success of penicillin and related compounds (β-lactam antibiotics i.e. first and third generation of antibiotics), which are presently the most used antibacterial agents, rests on both high efficacy and specificity. The β-lactam antibiotics restrict in a particular way with the biosynthesis of the peptidoglycan (Ghuysen 1991). This major constituent of the bacterial cell wall forms a three-dimensional network that completely surrounds the bacterium and protects it from its own osmotic pressure (Frere and Joris 1985). The peptidoglycan is also an

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essential element in maintaining the shape and rigidity of the cell wall. The physiological targets of β-lactam compounds are membrane DD-peptidases (often called penicillin-binding proteins), which are responsible for the synthesis and remodeling of the peptidoglycan. The β-lactam antibiotic acylate the active-site serine residue of the DD-peptidases, forming stable covalent non-catalytic acyl-enzymes. This results in the formation of non-functional peptidoglycan and, eventually cell death (Frere and Joris 1985, Jamin *et al.* 1995).

Like many other developing countries, in Bangladesh the use of antibiotics for treating human and animals is unregulated; antibiotics can be purchased in pharmacies, general stores, and even market stalls. This situation made alarming by the fact that antimicrobial drugs are often self-prescribed or by rural medical practitioners (barefoot doctors) with hardly any medical science knowledge (Mamun *et al.* 2006). This widespread use and misuse of antibiotics have resulted in resistance phenomena that have recently become increasingly widespread and worrying. As far the drug resistance is concerned, the β-lactamases are the main cause of bacterial resistance to penicillins, cephalosporins and related β-lactam compounds. These enzymes catalyze very efficiently the irreversible hydrolysis of the amide bond of the β-lactam ring, thus yielding biologically inactive product (Frere 1995). β-lactamases, which can be chromosome or plasmid encoded and produced in a constitutive or inducible manner, are secreted into the periplasmic space of Gram-negative strains or into the outer medium of their Gram-positive counterparts. However, due to unregulated use of antibiotics over the decades bacterial pathogenicity turned complicated by the genetic fluidity of microbial populations, which allowed a widespread and frightening distribution of β-lactamases plasmid genes (Richmond 1983, Davies 1994, Jacoby 1994). This evolution of β-lactamases also occurred by single-point mutations in β-lactamase-coding genes, resulting in the production of an ever-expanding number of enzymes with new substrate profiles (Payne and Amyes 1991, Bush *et al.* 1995). In the past decade, this happened extensively, especially due to abusive clinical utilization of antimicrobial drugs, which is responsible for the appearance of an increasing number of resistant strains. In most cases, this attributed to the production of new extended-spectrum β- lactamases (ESBL) (Philippon *et al.* 1989, Collatz et al 1990, Payne and Amyes 1991, Jacoby 1994). These plasmid-mediated enzymes confer resistance to β-lactamase-stable compounds such as cefotaxime, ceftazidime and aztreonam, all characterized by beta-acyl side-chains containing an oximino group. However, specific ESBL are selectively sensitive to specific β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam. These compounds, which have generally little antibiotic activity by themselves, behave as mechanism-based inactivators of most class of β-lactamase, and therefore are able to potentiate the action of classical β-lactamase sensitive compounds by protecting them from enzymatic hydrolysis. These drugs have been widely used against the β-lactamase producing bacteria and, for instance, clavulanic acid combined with amoxicillin seemed to be a powerful clinical strategy to overcome the resistance of bacteria harboring the β-lactamase variants (Seetulsingh *et al.* 1991).

However, bacterial susceptibility to such combinations of efficient β-lactamase antibiotics and potent β-lactamase inhibitor is now being challenged by the over production of β-lactamase (Martinez *et al.* 1989, Reguera *et al.* 1991, Seetulsingh *et al.* 1991). Moreover, recent reports also show presence of novel type β-lactamases in clinical isolates (Martinez *et al.* 2012, Mc Gann *et al.* 2012, Salabi *et al.* 2012), which are resistant to inhibitors. The inhibitor-resistant β–lactamases differ by one, two or three amino acids substitutions at their functional domain (Manageiro *et al.* 2012, Nordmann *et al.* 2012, Rodriguez-Martinez *et al.* 2012) that decrease the affinity for β-lactam substrates and alter the inhibitory action. Commonly these amino acid substitutions are located at the 69 (Methionine), 165 (Tryptophan), 244 (Arginine), 275 (Arginine) and 276 (Asparagine) position of the enzyme. This means that particular structural changes in the enzyme structures might modify their catalytic properties. However, despite the many available kinetic, structural and mutagenesis data, the factors explaining the diversity of the specificity profiles of β-lactamases of and their amazing catalytic efficiency have not been thoroughly elucidated. Nonetheless, recent identification of the

New Delhi metallo-β-lactamase-1 (NDM-$_1$) has led to international alarm, as its increase represents a new and important challenge in the field of infectious diseases (Kumarasamy *et al.* 2010). New delhi metallo-β-lactamases are enzymes that mediate resistance to various β-lactam agents, including carbapenems. NDM-$_1$ enzyme was named New Delhi after it had been originally first isolated from Sweden, when a NDM-1-positive *K pneumoniae* isolate was recovered from a patient who was an Australian resident of Indian origin and had visited Punjab in late 2009 (Yong *et al.* 2009). Since then, NDM-$_1$-producing organisms have been reported in hospitalized patients over the world. For this reason, in this research work we have evaluated the β-lactamase efficiency against several antibiotics that are regularly used in clinical practice. Our data shows that due to extinct use of penicillin, functional efficiency of beta-lactamases against it has eloped in our clinical isolates.

Materials and Methods

Sample collection and biochemical test for Bacterial identification: Samples were collected from surgery ward of Rajshahi Medical College Hospital. In this regard we chose the patients with post surgical wound infection but no improvement after β-lactam antibiotic treatment. Collected samples were identified using biochemical tests. Out of nine clinical isolates, five of them were β-lactamase secreting strains in acidometric test. Further biochemical test suggests that two of them are *E. coli*, one *Acinetobacter sp.*, one *Shigella sp.* and one *K. pneumoniae*.

Beta-lactamase efficiency test against different antibiotics: This was carried out according to Chen *et al.* (1984). Bacterial strains were grown in LB media overnight. Fifty μg/μl of each β-lactam antibiotic were separately placed in a microcentrifuge tube. Approximately 100 μl of overnight culture was dispensed in each substrate by brief agitation on a vortex apparatus, and incubated for 1 h at 37°C. After incubation, the tubes were centrifuged in a microfuge for 1 min to remove bacterial cells. Supernatant fluid from each tube was applied separately onto Whatman 3MM paper and heated at 120°C in an oven for 5 min. The fluorescent intensity of each test spot was then compared with its uninoculated substrate control spot under a long-wave UV lamp and classified as negative, weakly positive, or positive.

PCR detection of NDM$_1$ gene: Isolates were screened for NDM1 by PCR with primers NDM-$_1$F: 5'-CTTCCAACGGTTTGATCGTC-3' and NDM-$_1$R: 5'-TAGTGCTCAGTGTCGGCATC-3'. Amplified PCR products (465 bp) were separated on 1% agarose gel and visualized under UV.

Results

In order to verify the efficiency of β-lactamases secreting from different clinical isolates, rapid fluorescence end product spot test method was used. In this method, β-lactamase hydrolyzes the β-lactam antibiotic to open β-lactam ring. After heating it to 120°C hydrolyzed end product produces fluorescence. Interestingly this fluorescence is generated from β-lactam antibiotics only after β-lactamase hydrolyzation. However, spot test for antibiotic efficiency shows that apart from *K. pneumonia* none of the other clinical isolates are capable of hydrolyzing the penicillin. Isolated strains that were capable of hydrolyzing antibiotics, generated fluorescence spots under UV light. Here in this experiment as a negative control *E. coli* lab strain DH5-alpha was used, which is not resistant to any antibiotic and doesn't generate any fluorescence because of no β-lactamase was present (Fig.1 A). β-lactamases suppose to hydrolyze penicillin by default but due extinct use of it β-lactamases are more efficient against amoxicillin. We presume that this is due to changes in the functional domain of β-lactamases. Interestingly, one of the *E. coli* produces ESBL type 1 because this sub class of the β-lactamase is not inhibited by clavulanic acid. PCR analysis shows that *E. coli* that has resistance against clavulanic acid has NDM$_1$ gene expression (Fig. 1 B).

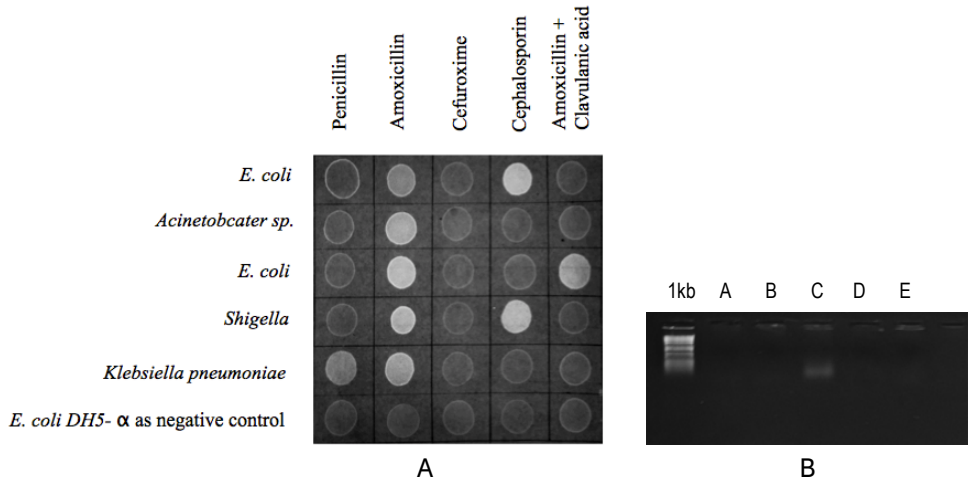

Fig. 1. A. β –lactamase efficiency detection by hydrolyzed antibiotics generated fluorescence. B. Identification of NDM1 gene using PCR amplification. NDM1 specific primers were used to amplify the NDM1 gene from our clinical isolates. Amplified PCR products (425 bp) were separated in a 1% agarose gel. Clinical isolates are sequencially A to E are *E. coli*, *Acinetobacter sp. E. coli*, *Shigella sp.* and *K. pneumonia*.

Discussion

Bacteria themselves are rapidly evolving organism and the rate of evolution comes to a critical consideration once several negative selection pressures work up on it. However, in our country, the unregulated use of antibiotic in fact creating a selection pressure on the bacterial population towards a pool of organisms with high pathogenicity. Since the discovery of penicillin bacteria have become efficient in escaping the lethality of β-lactam antibiotics by producing β-lactamases. Due to emergence of penicillinases bacteria, led to the development of cephalosporin β-lactam antibiotics, but production of plasmid-mediated ESBA (cephalosporinases) resulted in resistance to this drug class (Ripoll *et al.* 2011, Pathak *et al.* 2012). In addition to ESBA, NDM-$_1$ has added extra burden to the consequence (Bush and Jacoby 2010). However, in this research work we intended to understand the functional efficiency of β-lactamases against β-lactam antibiotics on a few of our local clinical isolates.

Our preliminary observation (Fig. 1) shows that out of five β-lactamases secreting strains are variably capable of defending β-lactam antibiotics. Presence of ESBL in the present findings implies that there might be plenty of these sorts of ESBL secreting strains that must be identified routinely and antibiotic recommendations should be according to culture sensitivity test. However, scientifically the most fascinating fact of our finding is that ideally the β-lactamase was supposed to hydrolyze penicillin by default but in our isolates secreting β-lactamase is not able to hydrolyze penicillin. In our clinical practice, use of original β-lactam like antibiotic penicillin has been stopped long before, instead its derivative amoxicillin is in sell in the market. Although our isolated β-lactamases are resistant against amoxicillin but couldn't degrade penicillin. We presume that some of the amino acids in the functional domain of β-lactamase have been changed respective to extinct use of penicillin. Mutations can impact the function of a protein through either direct or indirect mechanism. The direct means can be among the most obvious and involve the gain or loss of function. Mutation in the substrate binding pocket of the enzyme may change the affinity of substrate (Singh and Dominy 2012). For example, wild-type β-lactamase are not able to bind with cefotaxime to its binding pocket with high affinity but structural changes within the active site upon mutation have been suggested for developing resistance against cefotaxime (Huletsky *et al.* 1993, Cantu and Palzkill 1998).

A recent editorial by Ghafur (2010) highlights the widespread non-prescription use of antibiotics in India, leading to huge selection pressure, and predicts that the NDM-1 problem is likely to get substantially worse in the foreseeable future. This scenario is of great concern because there are few new anti-Gram-negative antibiotics in the pharmaceutical pipeline and none that are active against NDM-1 producers (Livermore 2009). Potential increase of NDM_1 among bacterial populations is a reason to be concerned (Bush and Jacoby 2010, Docobo-Perez *et al.* 2012, Zhang 2010). Besides our beta-lactamase efficiency test, in addition we have checked for presence of NDM_1 secreting strains in our clinical isolates. It was found that one out five β-lactamase secreting strains belongs to NDM_1 class. Indeed this finding has aroused the authenticity of further systematic search of β-lactamase in details for a better therapy. Moreover to achieve a better health system, knowledge on the size of the problem and early warnings of the emergence of resistant isolates are prerequisites. In Bangladesh laboratory diagnostic facilities are scarce with resultant introduction of empiric, pragmatic, and problem oriented management strategies for the administration of antimicrobial drugs but we have to keep in mind that antimicrobial drugs are an important resource that must be conserved for future use.

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