Asian Journal of Chemistry; Vol. 23, No. 7 (2011), 3183-3186

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

An Efficient Assay to Screen β-Lactamase Inhibitors from Plant Extracts

DURRE SHAHWAR[1,*], MUHAMMAD AKMAL KHAN[1,*], MUHAMMAD SIDDIQUE[2], MUHAMMAD SAJJAD[2], ZAHEER-UD-DIN KHAN[3], MUHAMMAD AJAIB[3] and NAEEM AHMAD[1]

[1]Department of Chemistry, GC University, Lahore-54000, Pakistan
[2]School of Biological Sciences, University of the Punjab, Lahore-54000, Pakistan
[3]Department of Botany, GC University, Lahore-54000, Pakistan

*Corresponding author: E-mail: drdshahwar@yahoo.com; akmalkhan73@gmail.com

(*Received*: 12 November 2010; *Accepted*: 18 March 2011) AJC-9758

β-Lactam-antibiotics (BLAs), the most widely used group of antibiotics, have significantly served the humanity, since their discovery. Some bacteria (*e.g.*, methicillin resistant *Staphylococcus aureus*) have developed mutated penicillin-binding proteins/enzymes and thus developed resistance to almost all of the β-lactam-antibiotics. Thus rendering antibiotic industry; worth US $25 billion almost to death-bed. Addition of a β-lactamase (BLase) inhibitor to β-lactam-antibiotics has been partially successful in alleviating this resistance. Though synthetic β-lactamase-inhibitors are at the scene, but search for new inhibitors from natural sources is a sound cry of the time. Preliminary screening of crude plant extracts is almost impossible using reported microbiological and/or instrumental techniques due to coloured nature of extracts, limited availability of purified β-lactamase and other problems. Hence a new, easy, efficient, economical and versatile assay was developed and practiced successfully in our laboratory. Transformed *Escherichia coli* DH5α with pET21α, which has a gene for β-lactamase was used. Transformation was carried out by modified basic $CaCl_2$ method with plasmid DNA. Assay was performed in test tubes according to reported templates, modified and managed to nullify colour problems. About 250 plant extracts were screened and the new method was found satisfactory in all aspects. Hence, it is suitable for large-scale screening of crude and semi-purified extracts.

Key Words: β-Lactam antibiotics, β-Lactamase, *E. coli* (DH5α), pET21α, Ampicillin resistant, Competent cells, Transformation.

INTRODUCTION

β-Lactam antibiotics (BLAs), like penicillin derivatives (penams), cephalosporins (cephems), monobactams and carbapenems, constitute one of the most important families of antibiotics[1,2]. They are essentially characterized by a β-lactam (BL) ring in their molecule and work by attacking the cell walls of bacteria. β-Lactam antibiotics acylate the active site serines of penicilline-binding proteins (PBP): a reaction that deprives bacteria of their physiological function and kills them[2-4]. But due to overuse in humans and as growth promoters in food of animals[5] some bacteria (*e.g.*, methicillin resistant *Staphylococcus aureus*; MRSA) have developed resistance to β-lactam antibiotics by several mechanisms and contributing factors. However, resistance by synthesizing β-lactamase (BLase); a group of enzymes that attacks and hydrolyzes the β-lactam-ring[6-8], continues to be the leading cause of resistance in gram-negative bacteria[9]. This resistance to β-lactam antibiotics, has emerged as a serious clinical problem over the last five decades and strains of bacteria that produce extended-spectrum-β-lactamases (ESBLases) have become very common[10,11]. These enzymes make BLAs ineffective as therapeutic agents. Extended-spectrum β-lactamases-producing bacterial strains are highly resistant to an array of antibiotics so infections by these strains are difficult to treat. Thus, the choice of effective and safe drugs to be used is shrinking day by day[12].

Emergence of plasmid-mediated resistance raises question about the future of antibiotics in chemotherapy, as the transfer of such resistance-plasmid to other bacteria will promote the fast spread of resistance genes[13]. According to a recent study about *E. coli*, the rate of adaptative-mutations is on the order of 10^{-5} per genome per generation, which is thousand times as high as previous estimates[14]. As an outcome of extensive microbiological, biochemical and genetic investigations more than 270 β-lactamases have been described and divided into four molecular classes: A, B, C and D[15].

Although BLase-related resistance can be approached using multiple therapeutic interventions including non-BLAs, BLase-stable-antibiotics or BLase-inhibitors. Idea of antibiotic

combinations containing the BLasé-inhibitors seems the most convenient and a novel clinical approach to control ESBLase-resistant organisms[4]. However, the current marketed inhibitors (tazobactam, clavulanate and sulbactam) are not active against all β-lactamase. These combinations inhibit only the growth of bacteria producing molecular class-A-BLases, such as the common TEM (Temoniera) and SHV (sulphydryl variable), which are often encoded by plasmids in gram-negative bacteria. They do not show reasonable activity against the class-C (chromosomal serine cephalosporinases) or the metallo-BLases (class-D) that are now appearing more frequently on multi drug resistant plasmids[16]. Moreover, in the early 1990s BLases, that were resistant to clavulanic acid were also discovered[2,17]. Therefore, development of synthetic and/or discovery of natural BLase inhibitors to be combined with BLAs, to combat infectious diseases, caused by multidrug-resistant-bacteria (MRB) including fast-spreading, ESBLase-producing enteric bacteria, is a sound cry of time. Therefore, due attention is needed to develop new alternative or combination agents[12,17].

A large-scale search for BLase inhibitors requires rapid, sensitive and chromogenic assays. A reasonable number of microbiological and/or instrumental assays/procedures have been reported.

Starch paper method that uses discoloration of iodine[18].

Starch-iodine agar plate method[17].

Iodometric method In-Tube[19] and by Catlin-method[20].

Acidimetric method using paper discs and phenol red as indicator[21].

Chromogenic cephalosporin method: employing a commercial reagent; nitrocefin, prepared as liquid solution[22].

Inhibition method using chocolate agar plates[23].

Reverse passive haemaglutination procedures[7,24,25].

Tube broth dilution method, using specific dye (*p*-iodonitro tetrazolium violet)[26].

The nitrocefin competition assay[27] which is widely used to screen for BLase-inhibitors from plant extracts.

Nitrocefin is a chromogenic BLase substrate that undergoes distinctive colour change from yellow (λ_{max} 390 nm) to deep red (λ_{max} 486 nm) as the amide bond in the BL-ring is hydrolyzed by Blasé[28]. These characteristics are suitable for convenient detection of BLase activities. Nitrocefin is commercially available but is prohibitively expensive (*ca.* $20,000/g) because of the circuitous routes to its synthesis[29]. Though it has been used in competitive inhibiton studies in development work on BLase-resistant antibiotics using Direct plate method, Slide method (avoid dessication), Broth method, Broken cell method, Paper disc spot method or spectrophtometric assay. But while working with nitrocefin one has to protect it from light to avoid degradation and store at -20 ºC. Stock solution could be stored at -20 ºC for only up to 2 weeks[30]. Yet preliminary screening of crude plant extracts is almost impossible using reported techniques. It is because the solution of the extracts is often brown, green or yellow and hinders the reasonable judgment of screening experiments, more over limited availability of purified BLase at large scale is another problem. Hence a new, easy, efficient, economical and versatile assay was developed and practiced successfully in our laboratory.

EXPERIMENTAL

Transformation of bacteria: In this study a laboratory strain of *E. coli i.e.*, DH5α transformed with pET-21α plasmid; which has a gene for BLase was used. Transformation was carried out according to modified Russel and Sambrook's protocol for molecular cloning[31].

Solutions and media for 250 mL: $CaCl_2 \cdot 2H_2O$, 1.83 g (= 50 mM). Autoclaved and stored at 4 ºC. LB medium and LB plates supplemented with appropriate antibiotic to select for the plasmid (ampicillin in this case, at 100 µg/mL, final concentration).

Preparation of competent cells: Set up overnight (16 h) culture (5 mL) of the host strain at 37 ºC with shaking. Next day, inoculate the overnight culture (0.2 mL) into fresh pre warmed LB (10 mL in a 100 mL flask) and incubate with shaking at 37 ºC until the A_{550} of the culture is *ca*. 0.2-0.3 (this normally takes about 2 h). Place the culture on ice for 5 min then transfer it to a pre cooled (4 ºC) sterile oakridge centrifuge tube. Spin the cells down (in the Beckman J2-21) using a pre cooled roter at 6000 rpm for 5 min at 4 ºC. Discard the supernatant. Gently re suspend the cells in ice-cold 50 mM $CaCl_2$ (*ca*. 20 mL) and leave on ice for 40 min. Repeat last step. Discard the supernatant. Gently re-suspend the cells in ice-cold $CaCl_2$ (2 mL) and store on ice until needed. These are the competent cells. Competent cells are checked by streaking on the lauria bertin + ampicllin plates.

Transformation of cells and plating out: Dispense a sample of the competent cells (200 µL) into a sterile pre cooled micro centrifuge tube. Add the plasmid DNA; mix gently and leave on ice for 40 min. Quickly transfer the tube to a 42 ºC water bath for 2 min then return it to ice for *ca*. 5 min. Add LB medium (0.8 mL) to the tube, mix and incubate it for 1-2 h at 37 ºC without shaking. Spread samples of the transformed cells (50-200 µL) on pre warmed, dried LB plates containing the appropriate antibiotic (ampicillin in this case) to select for the plasmid.

Plant extracts: Over a period of *ca.* 2 years more than 250 plant extracts were extracted/ arranged. A variety of plant extracts including essential oils as well as soaked extracts from different parts of plants, trees, shrubs and herbs from many families found in Pakistan were used. The following extraction techniques/schemes were employed: Essential oils by expression, enfleurage, maceration, solvent extraction and steam/ hydro distillation with Clevenger type apparatus and later on recovered with diethyl ether and dried with anhydrous sodium sulphate[32]. Sequential extraction with petroleum ether, chloroform, ethyl acetate, acetone, methanol, ethanol, *n*-butanol and water using soxhlet extractor assembly[33]. Solvent extraction/ fractionation of soaked methanolic/ethanolic extracts using separating funnel according to general reported procedures[34].

Antibacterial activity: Assay was performed in test tubes (TTs) according to the procedure developed and standardized in our laboratory. Actually the method originated from reported templates with Muller-Hinton broth (MHB), lauria bertin (LB) and nutrient broth (NB), *etc*., based antimicrobial assays including disc diffusion method[35], agar-well diffusion method[34,36], macro and micro dilution assays *etc*.[37-41] modified and managed to nullify the colour, concentration and solubility problems:

TABLE-1
CLEARLY ILLUSTRATES OBSERVATIONS DURING WORK AND THUS INFERENCES DERIVED

TT#	Contents of TT	Observation (4-18 h)	Inference/Result
1	N. Broth + Plant extract + Ampicillin	No change as expected.	It served as a colour reference for comparison (*i.e.* –ve control) of experimental samples
2	N. Broth + Ampicillin *E. coli* (DH5α) inoculum	TT contents turned turbid due to growth of bacteria	Bacterial strain is ampicillin resistant through Blasé production (*i.e.* +ve control)
3	N. Broth + Plant extract + *E. coli* (DH5α) inoculum	1. TT contents turned turbid as compared with TT#-1 due to growth of bacteria 2. TT contents show no turbidity as compared with TT#-1 *i.e.* no growth of bacteria	1. Plant extract is not antibacterial in its own (*i.e.* +ve control). 2. Plant extract is itself effective against BL resistant mutant strain through some other mechanism
4	N. Broth + Plant extract + Ampicillin + *E. coli* (DH5α) inoculum	1. TT contents turned turbid as compared with TT#-1 due to growth of bacteria 2. TT contents: no turbidity as compared with TT# 1 *i.e.* no growth of bacteria	1. Plant extract is not BLase inhibitor. 2. Plant extract is Blasé-inhibitor

Ampicillin and extract solutions were sterilized by micro-filtration with syringe-filters (20 µm, PTFE, Starlab Scientific Co. Ltd.) into sterilized/autoclaved vials while working in the Laminar Flow Cabinet. 3 mL LB solution (27 g/dm^3) was added to wider (38 mm × 200 mm) culture/test tubes (TTs), cotton plugged and autoclaved (15-20 min, 121 ºC, 0.11 MPa). After cooling to room temperature, according to scheme given in Table-1, 500 µL of each of sterilized plant extract solution (16 mg/mL) and ampicillin solution (16 mg/mL) were added to make total volume 4 mL and hence get final concentration of 2 mg/mL each. For positive/negative controls water and/or relevant solvent were added to make-up the volume. Plugged TTs were put on rotary shaker (*ca.* 50-100 rpm) to homogenize for 15-30 min. 15-18 h old inoculum was adjusted/diluted to $10^{6\text{-}8}$ CFU with sterile saline solution by spectrophotometry (530 nm) corresponding to 0.5 McFarland standard[42]. Then 20 µL of inoculum were added to each TT, while working in the laminar flow cabinet. Put on rotary shaker (100-150 rpm) to assure homogeneity and to maintain aerobic conditions as well. $HgCl_2$[18], Clavualnic acid and Augmentin (injection) were used as standard inhibitors. Observed during 4-18 h any changes in turbidity/colour and reported according to Table-1.

In another study for kinetics the turbidity growth rate was monitored spectrophotometrically. At regular intervals 20 µL samples were drawn and immediately diluted with acetone and water to stop any further bacterial growth as well as to adjust optical density (λ_{max} 500-600 nm).

RESULTS AND DISCUSSION

About 250 plant extracts were screened and the new method was found satisfactory in all aspects. Hence it is suitable for large-scale screening of purified, semi purified as well as crude extracts.

Advantages, versatility and cares: The method is equally applicable to all types of extracts, regardless of their colour, concentration, impurity content and to some extent solubility. A variety of bacterial strains (both natural and transformed) could be screened, safely and cheaply for their susceptibility towards extracts, because it is only once you have to have bacteria and then through frequent sub culturing it is possible to go ahead without further investment. The bacteria can be tuned through proper choice of plasmid to produce either of the BLases: A, B, C and D or other PBPs. Not only that but other resistant strains (*e.g.*, kanamycin reistant) could also be screened through required transformation with appropriate plasmid. The method is applicable for all bacterial strains which can host plasmids like pET21-series, pTZ57-series *etc*. The method is free of possible confusions encountered with other methods due to factors like restricted diffusion of enzyme and extract solutions in agar, denaturation of enzyme during purification, decolourization of iodine by salicylic acid or other compounds and requirement of fresh plates *etc*.[18]. *E. coli* under stress readily transfers multidrug resistant plasmids to other species of bacteria, such as *Staphylococcus aureus*, through a process called horizontal gene transfer[14] so the safer lab strain of *E. coli* should be avoided to come in contact with other natural strains.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Ahmad Adnan and Miss Zareena Mushtaq, Process Development Laboratory, Department of Chemistry, GC University, Lahore, for their valuable suggestions and support.

REFERENCES

1. D.M. Livermore, *J. Infect. Dis. Soc*., **6**, 75 (1995).
2. J.G. Piéboji1, S. Baurin, J.M. Frère, P. Ngassam, B. Ngameni, A. Azebaze, D.E. Pegnyemb, J. Watchueng, C. Goffin and M. Galleni, *Phytother. Res.*, **21**, 284 (2007).
3. K.B. Holten and E.M. Onusko, *Am. Family Phys*., **62**, 611 (2000).
4. K. Bush and S. Mobashery, *Adv. Exp. Med. Biol*., **456**, 71 (1998).
5. J. Johnson, M. Kuskowski, M. Menard, A. Gajewski, M. Xercavins and J. Garau, *J. Infect. Dis*., **194**, 71 (2006).
6. M.H. Richmond and R.B. Sykes, *Adv. Microb. Physiol*., **9**, 31 (1973).
7. D.M. Livermore, *Clin. Microbiol. Rev*., **8**, 557 (1995).
8. M.H. Nicolas-Chanoine, *Int. J. Antimicrob. Agents*, **7**, S21 (1996).
9. P.A. Bradford, *Clin. Microbiol. Rev*., **14**, 933 (2001).
10. D.L. Paterson and R.A. Bonomo, *Clin. Microbiol. Rev*., **18**, 657 (2005).
11. M.E. Jones, D.C. Draghi, C. Thornsberry, J.A. Karlowsky, D.F. Sahm and R.P. Wenzel, *Ann. Clin. Microbiol. Antimicrob*., **3**, 14 (2004).
12. I. Ahmad and F. Aqil, *Microbiol. Res*., **162**, 264 (2007).
13. K.L. Hopkins, R.H. Davies and E.J. Threlfall, *Int. J. Antimicrob. Agents*, **25**, 358 (2005).
14. http://www.sciencemag.org/cgi/content/abstract/317/5839/813?maxtoshow=&HITS=10&hits=10&RESULTFORMAT=&fulltext=isabel+ gordo&searchid=1&FIRSTINDEX=0&resourcetype=HWCIT
15. R.P. Ambler, *Philos Trans R. Soc. Lond. Biol. Sci*., **289**, 321 (1980).
16. Y. Doi, Ji. Wachino, M. Ishiguro, H. Kurokawa, K. Yamane, N. Shibata, K. Shibayama, K. Yokoyama, H. Kato, T. Yagi and Y. Arakawa, *Antimicrob. Agents Chemother*., **48**, 2652 (2004).
17. Y. Zaichang, Y. Xaosheng and N. Yule, *Int. J. Integ. Biol*., **6**, 62 (2009).
18. T.O. Odugbemi, S. Hafiz and M.G. NcEntegrat, *Brit. Med. J*., **2**, 5001 (1977).

19. C. Thornsberry, Laboratory Training and Consultation Division Services, Centers for Disease Control, pp. 1-8 (1980).
20. B.W. Catlin, *Antimicrob. Agents Chemother.*, **217**, 1 (1975).
21. C. Thornsberry and L.A. Kirven, *Antimicrob. Agents Chemother*., **6**, 653 (1974).
22. R. Llanes, J. Sosa and I. Martínez, *Sex Transm. Inf*., **76**, 58 (2000).
23. H.M. Palmer, J.P. Leeming and A. Turner, *J. Antimicrob. Chemother*., **45**, 777 (2000).
24. W. Hodge, J. Ciak and E.C. Tramont, *J. Clin. Microbiol*., **7**, 102 (1978).
25. T.R. Oberhofer and D.W. Towle, *J. Clin. Microbiol*., **15**, 196 (1982).
26. J.N. Eloff, *Planta Med*., **64**, 711 (1998).
27. G.A. Papanicolaou and A.A. Medeiros, *Antimicrob. Agents Chemother*., **34**, 2184 (1990).
28. C.H. O'Callaghan, A. Morris, S.M. Kirby and A.H. Shingler, *Antimicrob. Agents Chemother*., **1**, 283 (1972).
29. M. Lee, D. Hesek and S. Mobashery, *J. Org. Chem*., **70**, 367 (2005).
30. Calbiochem; data sheet 48440 Rev. 10-January-06 RFH. Nitrocefin Cat. No. 484400
31. J. Sambrook and D.W. Russell, Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (2001).
32. J. Lawless, The Illustrated Encyclopedia of Essential Oils: The Complete Guide to the Use of Oils in Aromatherapy and Herbalism (ISBN 1852307218) (1995).
33. M.A. Khan, D. Shahwar, N. Ahmad, Z.D. Khan and M. Ajaib, *Asian J. Chem*., **22**, 379 (2010).
34. I. Ahmad and A.Z. Beg, *J. Ethnopharmacol*., **74**, 113 (2001).
35. A.W. Bauer, W.M.M. Kirby, J.C. Sherris and M. Turch, *Am. J. Clin. Pathol*., **45**, 494 (1996).
36. C. Perez, M. Pauli and P. Bazerque, *Acta Biol. Med. Exp*., **15**, 113 (1990).
37. National Committee For Clinical Laboratory Standards (NCCLS-USA), Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard M27-A, Wayne, Pa; National Committee for Clinical Laboratory Standards, **17**, 1 (1997).
38. National Committee for Clinical Laboratory Standards, Performance Standards for Antimicrobial Disk Susceptibility Tests, Approved Standard-A5, National Committee for Clinical Laboratory Standards, Villanova, PA, edn. 5 (1993).
39. J.B. Casals and N. Pringler, Antibacterial/Antifungal Sensitivity Testing Using Neo-Sensitabs, Rosco Diagnostica, Taastrup, Denmark, pp. 55-58 (1991).
40. I. Karaman, F. Sahin, M. Gulluce, H. Ogutcu, M. Sengul and A. Adiguzel, *J. Ethnopharmacol*., **85**, 231 (2003).
41. National Committee for Clinical Laboratory Standards (NCCLS), Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Proposed Standard, NCCLS Document M31-A, Villanova, PA (1998).
42. L.M.F. Soni, M. Burattini, A. Pignatari, O. Gompertz and A. Colombo, *Mycopathologia*, **145**, 131 (1999).