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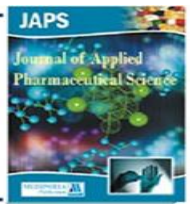

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Studies on extended beta lactamase producing, biofilm forming clinical bacterial pathogens and its invitro inhibition by Actinobacterial extracts

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ABSTRACT

At present scenario, the extended spectrum beta lactamase (ESBL) producing bacterial pathogen causes various life threatening infections especially by the members of the family Enterobacteriaceae in hospital settings. In order to study the prevalence of ESBLs in Kanchipuram hospital, the bacterial strains were isolated from patients having Urinary tract infections (UTI), diabetic foot ulcer, pregnancy women's, surgical wound infections, deep wounds, and genitourinary tract problems. Totally 40 bacterial isolates were recovered from 30 samples and the isolates were identified as *Escherichia coli* (45%), *Pseudomonas sp*, (25%) *and Klebsiella sp* (30%). The ESBL production was confirmed with third generation cephalosporins (cetixime, cephoxitin, ceftazidime, cetepime, ceftriaxone, ceftazidime/clavulanic acid) using the Kirby- bauer disc diffusion method and also by double disc diffusion method. The highest ESBL production was found among *E. coli* (42%), followed by *Pseudomonas sp*. (25%) and *Klebsiella sp* (20%). All the ESBL producer were tested for biofilm formation by tube method in which *E.coli* (43%) was found to be the good biofilm producer followed by the *Klebsiella sp* (31%) and *Pseudomonas sp* (25%). An attempt was also made to study the *in-vitro* inhibition of biofilm forming ESBL pathogens by actinobacterial extracts by disc diffusion method. Of the five actinobacterial extracts tested, extracts produced from the strain MA7 inhibited (8-12 mm zone of inhibition) all the biofilm forming ESBL pathogens. Further purification and characterisation of active compound from actinobacterial strain MA7 is in progress.

Key words: bacterial pathogens, ESBL production, biofilm, actinobacterial extracts.

INTRODUCTION

The widespread use of antibiotics both inside and outside of medicine is playing a significant role in the emergence of resistant bacteria. Antibiotic resistance is a type of drug resistance where a microorganism is able to survive exposure to an antibiotic. Certain antibiotic classes are highly associated with colonization of superbugs (Goossens et al, 2005; Motta et al, 2003). Now days, about 70% of the bacteria that cause infections in hospitals are resistant to at least one of the drugs most commonly used for treatment. Some organisms are resistant to all approved antibiotics and they can only treat with experimental and potentially toxic drugs (Todar et al, 2008). The extended - spectrum β-lactamases (ESBLs) are mutant, plasmid mediated β-lactamases which are derived from the older, broad spectrum β-lactamases. ESBLs have spread threateningly in many regions of the world and they presently comprise over 300 variants. ESBL producing microorganisms are very dynamic and constitutes an increasing problem due to their

hydrolyzing activity against extended spectrum third generation cephalosporins, such as cefotaxime, ceftriaxone, ceftazidime and the monobactam aztreonam often employed in the treatment of hospital acquired infections (Romero et al, 2005; Dechen et al, 2009). The majority of ESBL producing strains are *Enterobacteriacea*e members such as *Klebsiella pneumoniae, Klebsiella oxytoca* and *Escherichia coli* (Ami et al, 2008).

There is now widespread recognition of biofilms to human infection. Previously thought to be the concern only of industrial and environmental microbiologist interested in phenomena such as biofouling, it is now clear that microbial biofilms are largely responsible for the recalcitrance of many infections to conventional antimicrobial therapy. Microbes attached to a surface are usually considered to be less susceptible to antimicrobial agents and are protected from the host immune response, giving rise to chronic infections that are notoriously difficult to eradicate (Lewis et al, 2007). By forming a matrix encased multicellular aggregate cells can also escape from engulfment by phagocytic cells. Surprisingly little is known about the inhibitors of biofilm forming ESBL pathogens. Among the various industrially important microorganisms, actinobacteria are of prime importance and are primarily recognizes as organisms of academic curiosity and also as potential antibiotic producers. With this view the present study was initiated to study the ESBL producer with biofilm forming ability of clinical pathogens in Kanchipuram, Tamilnadu and also to exploit actinobacteria against these pathogens.

MATERIALS AND METHODS

Collection of samples

Thirty samples were collected for a period of 3 months between 17-10-2010 to 25-12-2010 from the patients having Urinary tract infections (UTI), Diabetic foot ulcer, Pregnancy women's, Surgical wound infections, deep wounds, and genitourinary problems by using sterile swabs in hospitals situated at Kanchipuram. All the samples were aseptically transported in nutrient broth within 45 minutes of sample collection.

Isolation, identification and antibiotic susceptibility testing

The collected samples were inoculated into nutrient agar plates and incubated at 37°C for 24 hours. Totally 40 morphologically different isolates were selected from isolation agar. The isolates were plated in media such as Eosin Methylene Blue agar, Cetrimide agar, and MacConkey agar. The plates were incubated at 37°C for 24 hours. The colonies with characteristic growth were subjected to routine micromorphological test such as gram staining, capsule staining, spore staining, motility, and biochemical tests such as Catalase, oxidase, IMViC, TSI, carbohydrate utilization test, urease. All the isolates that were identified were subjected to antimicrobial susceptibility test by Kirby Bauer disk diffusion assay using third generation cephalosporins antibiotic disc such as Cefixime, Cephoxitin, Ceftriaxone, Cefepime, Meropenem, Imipenem, and Extapenem. The diameter of zone was measured and recorded to the nearest millimeter. The strains that were resistant to antibiotic in Kirby Bauer disk diffusion were selected for the detection of ESBL producers.

Screening and identification of extended spectrum beta lactamases (ESBL) producing strains (Double Disk Diffusion test)

The test inoculum (0.5 McFarland turbidity) was spread on MHA plates, by using sterile cotton swabs. A disc of Ceftazidime +clavulanic acid ($Cac^{30/10}$) was placed on the surface of MHA then disc of Ceftazidime (30µg/disc) was placed at a distance of 10mm apart from $Cac^{30/10}$ disc. The plates were incubated at 37°C for 24 hours. Enhancement of the zone of inhibition between the clavulanate disc and any one of the β lactam discs indicated the presence of an ESBL (Jain et al, 2003).

Detection of biofilm forming isolates

Hundred microlitre of test culture was taken and transferred in to test tubes containing 3ml of nutrient broth. The tubes were incubated at 37ºC for 24 hours. After incubation period, cultures was decanted from the tube and washed with 3 ml phosphate buffer saline (PBS-1X), and allowed to dry. Dried tubes were stained with 3 ml of 2% crystal violet solution and allowed for 5 minutes. The stain was discarded and the tubes were washed in running tap water. The formation of biofilm can be observed visually. Finally 1.5 ml of 33% glacial acetic acid was added and mixed thoroughly. Optical density (OD) was taken at 570nm (Teng et al, 2003).

Invitro inhibition of ESBL pathogens by actinobacterial extracts

Crude extracts prepared from five actinobacterial strains (MA7, BCA1, YA3, CA4, C3) were obtained from Department of Microbiology, Sri Sankara Arts and Science College. Antibacterial activity of crude extracts was tested against ESBL pathogens by disc diffusion method. 0.25 mg of crude extract was impregnated in sterile filter paper disc with 5 mm diameter. The test pathogens were inoculated into nutrient broth and incubated at 37°C for 18 hours. Each of the culture was adjusted to 0.5 McFarland turbidity and inoculated onto Muller Hinton agar (MHA) with the help of sterile swabs. The crude compound impregnated discs were placed over the test organisms. The diameter of the inhibition zone was measured after 24 hours of incubation at 37°C (Selvameenal et al, 2009).

RESULT AND DISCUSSION

Totally thirty number of samples were collected from hospitalized patients. Three different organisms were identified from the collected samples. There has been high incidence of *E.coli* (45%), *followed by K. pneumoniae* (30%), *and Pseudomonas sp* (25%). Similar to our study Abdulrahman et al, 2005 reported that common isolates from the clinical samples were E. *coli, Klebsiella sp* and *Pseudomonas sp*. Antibiotic resistance

Table.1: Antibiogram assay of isolated strain to third generation antibiotics.

S. No	Antibiotics	Percentage of resistance strains (n=16)
1	Cetixime	94
2	Cephoxitin	94
3	Cefrtiaxone	94
4	Cetepime	69
5	Meropenem	12.5
6	Imipendem	0
7	Extapenem	0

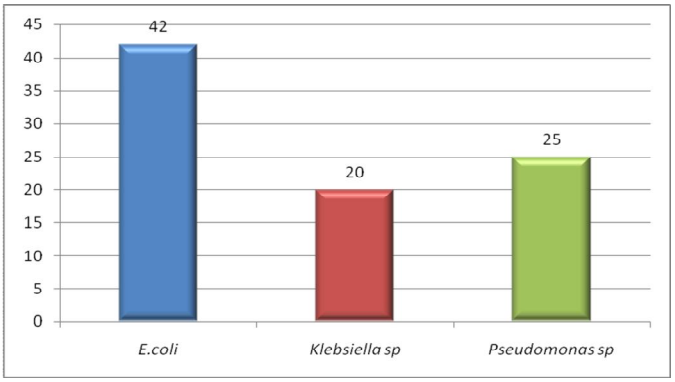

Fig. 1: Screening of drug resistance bacteria for ESBL producers by double disc diffusion method.

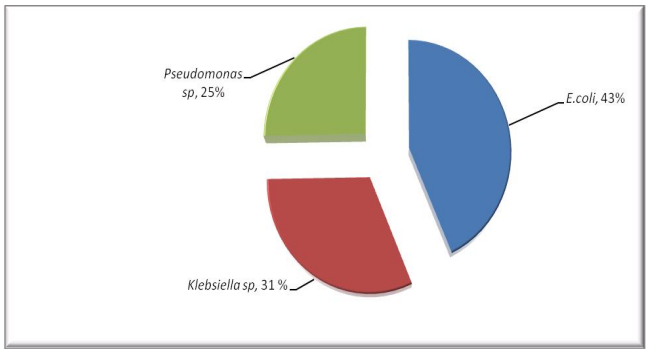

Fig.2: Percentage of biofilm forming ESBLs.

Table-2: In vitro inhibition of biofilm forming ESBL pathogens by actinobacterial extracts.

Bacterial Strains	Zone of inhibition in mm Actinomycete extracts				
	MA7	BCA 1	YA3	CA4	C3
U2	10	13	-	-	-
U4	12	-	12	-	-
U5	13	12	-	-	-
U10	8	-	10	-	-
U16	12	10	10	-	-

pattern of the isolated pathogens showed resistance to most of the third generation antibiotics. The isolates which showed resistance to most of the antibiotic were selected for testing ESBL producers. From the results of antibiotic sensitivity test it has been observed that the clinical isolates of *E.coli*, *Klebsiella sp*, and *Pseudomonas sp* were showing resistance to almost all the antibiotics tested expect to impendum and extrapenum. The extensive upsurge of antibiotic resistance is in accordance with an earlier report by Obseiki Ebor et al., 1987 where it was reported that antibiotic abuse and high prevalence of self medication with antibiotics are responsible for the emergence of antibiotic resistant bacterial strains. The prevalence of ESBL production was common among Enterobacteriaceae members (Huiwang et al, 2003) and ESBL occurrence was higher in the hospitalized patients (Anthony et al, 2007). The frequency of ESBL producers of 31.5% in our study is comparable to previous studies (Singhal et al, 2005). In the present study, the highest ESBL producer was *E. coli* (42%), followed by *Pseudomonas sp.*, (25%) and *Klebsiella sp* (20%) (Figure 1) This demonstrated that ESBL screening should not be limited only to *E.coli* and *Klebsiella sp.* The other species which showed ESBL production was *Pseudomonas sp.*

The ability to adhere to materials and to form biofilm is an important feature in the pathogenesis of clinical isolates. The present study noticed that ESBL producer of *E. coli* (43%) was the most potent biofilm forms followed by the *Klebsiella sp* (31%) and *Pseudomonas sp* (25%) (Figure 2). To date, only medicinal plants have been reported to inhibit biofilm formation (Limsuwan et al, 2008). The bioactive capabilities of actinobacteria have yet been explored and the present study studies show that actinobacteria may be a promising candidate with potential inhibitory activity against biofilm forming ESBL producing clinical pathogens. Among the five extracts tested MA7 showed inhibitory activity against the biofilm forming ESBL pathogens (Table 2).

CONCLUSION

The present study concludes that the prevalence of biofilm forming ESBL producers was found to be more in Kanchipuram that were not able to treat with the most of the available antibiotics. Hence, further studies on the efficacy of these actinobacterial extracts against ESBL producing biofilm forming clinical pathogenic organisms and characterization of the Compound, *in vivo* toxicological studies must be carried out with an outlook of developing more novel drugs for human welfare.

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