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Prevalence of ESBL pathogens in Salem Hospitals and its control

Aruna. S, S. Ramya and *R. Balagurunathan

Department of Microbiology, Periyar University, Salem, Tamil Nadu, India

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ABSTRACT

Organisms developing resistance against antimicrobial compound is the growing problem worldwide. Resistance mechanisms frequently found in every class of antibiotic agents. The detection and prevention of such resistant organisms are in major concern. With this view the present study is attempted to study the prevalence of ESBL pathogens in Salem district hospitals. Pus and urine sample collected from Salem district hospitals. The isolates are subjected for antibiotic sensitivity test by using cephalosporin antibiotic and Identified by biochemical test. ESBL resistant strains are phenotypically characterized by slime, haemolysin and beta lactamase production. Genotypic identification by using fim, pap and cnf genes for E.coli and alg-D gene for Pseudomonas. To find out the potent bioactive compound for controlling these pathogens, they are screened against six Streptomyces sp. extracts. From Fourty clinical samples morphologically distinct 53 strains of bacteria were isolated and identified as E.coli (45%), P.aeruginosa (42%), K.pneumoniae (18%) and S.aureus (40%). Four E.coli isolates and one P.aeruginosa showed resistant to antibiotic and confirmed as β-lactamase producers. In slime detection 3 E.coli and one P.aeruginosa strains showed strong positive results and 2 E.coli strains showed positive results for haemolysin. In case of beta lactamase all the isolates showed positive result. For E.coli 2 virulence genes pap (75%) and cnf (25%) are amplified and no isolates produced fim gene. 100% of algD virulence gene is amplified for Pseudomonas. Only two extracts Y1 and P7 have highest antagonistic activity against E.coli and P.aeruginosa. Conclusion: The results indicated that actinomycetes will play an essential role in protecting human health by producing novel antibiotics against resistant microorganisms.

Key words: ESBL, gram negative bacilli, virulence factors, actinomycetes, urinary tract infections.

INTRODUCTION

Nowadays ESBL producing microbes are emerging threat. The main mechanism for this ESBL resistance by production of enzyme called extended spectrum β-lactamases (ESBLs). The β lactamase enzymes produced by the organisms break down the structural beta-lactam ring of β-lactam antibiotics. Many genera of gram negative bacteria possess a naturally occurring, chromosomally mediated β-lactamase and also some are plasmid mediated β-lactamases [1]. Beta lactams family consisting of four major groups: penicillins, cephalosporins, monobactams and carbapenems and all group of antibiotics having β-lactam ring, which can be hydrolyzed by β-lactamase enzyme produced from bacteria. The enzyme contains serine at the active site which reacts mainly with the carbonyl carbon of the β-lactam ring leads to opening of the ring (inactive β lactam) and regenerating the β lactamase [2]. In the past two decades, gram negative bacilli producing ESBL causes major problem in hospital setup. Currently 70% of the infections caused by the bacteria in hospitals that are resistant to at least one of the drugs commonly used for routine treatment purposes [3]. ESBLs are enzymes which inactivate the penicillin, cephalosporin of the first, second and third generation antibiotics like cefotaxime, ceftazidime and the monobactam antibiotic aztreonam by inhibiting the β-lactam ring and inactivate those antibiotics. Such ESBL enzymes commonly found in *Klebsiella pneumoniae*,

Pseudomonas aeruginosa and *Escherichia coli* and also detected in *Klebsiella oxytoca, Proteus mirabilis, Salmonella* species and other members of *Enterobacteriaceae* [4]. Worldwide data shows that the ESBL resistance is increasing among UTI pathogens due to usage of conventional drugs. Occurrences of ESBL producing *Enterobacteriaceae* have also reported in South India and Central India [5]. Apart from cities ESBL pathogens spreads to the small town also. To prevent this potential emergence, a replacement of existing antibiotics is necessary. Actinomycetes are the best known for their ability to produce antibiotics [6]. The actinomycetes are Gram positive bacteria having high G+C (> 55%) content in their DNA. Of all known drugs 70% have been isolated from actinomycetes of which 75% and 65% are used in medicine and agriculture purposes [7]. They produce numerous bio-active compounds for health such as enzymes, antibiotics. This study aims to isolate and characterize the prevalence of ESBL producing pathogens in Salem hospital patients and its control by actinomycetes extract.

MATERIALS AND METHODS

Sample collection

This study was conducted during the period between December 2010 and June 2011. 25 urine samples and 15 pus samples were collected from patients suffered with wound infections and genitourinary problems by using sterile swabs and sterile disposable container in Gokulam hospital and SKS hospital, Salem (Latitude : 11° 41' N and Longitude : 78° 07' E), Tamil Nadu. The collected swabs were transferred in to nutrient broth within 45 minutes and brought to the laboratory.

Isolation and identification of microorganisms

The transported nutrient broth was incubated at 37^{o} C for 24 hours. After incubation, one loopful of culture was inoculated into nutrient agar plates for further studies. Morphologically distinct colonies were isolated and further streaked on the selective media plates like mannitol salt agar, eosin methylene blue and also in differential media such as macconkey agar and blood agar. The plates were incubated at 37^{o} C for 24 hours. After incubation period the plates were observed and identified for the bacterial strains. Identification of all isolates was done by routine biochemical tests according to Bergey's manual and the results were interpreted [8].

Antibiotic sensitivity test

The method recommended by Bauer 1966 was followed. The antibiotic sensitivity for each isolate was carried out on Mueller-Hinton agar by Kirby-Bauer disc diffusion technique. The antibiotics used were cephadroxil, cefoperazone, ceftazidime, cephotaxime and ciprofloxacin at 25mcg concentration per discs. The test organisms (*E.coli, K.pneumoniae, P.aeruginosa and S.aureas*) were uniformly lawn on the sterilized Mueller Hinton agar plates with the help of sterile swabs and left undisturbed for 30 minutes. The antibiotic discs were placed over the media and incubated at 37^{o}C for 24 hours. After incubation the zone of inhibition was measured and interpreted based on CLSI guidelines [10] for quality control of disc diffusion test [9].

Characterization of isolates by phenotypic and genotypic methods

There was several virulence factors were produced by the different microorganisms like *E.coli, P.aeruginosa*. Some common virulence factors were haemolysin production, slime production, beta lactamase in phenotypic method. *fim, pap, cnf* and *alg-D* virulence factor gene used for genotypic method by PCR.

Determination of virulence factors by phenotypic methods

Slime activity

BHI agar plates were prepared with 0.8 g/l Congo red. *E.coli* and *Pseudomonas aeruginosa* isolates were placed on the surface of the medium and incubated at 37^{o}C for 24 hours. Slime producing colonies was examined under obliquely reflected light on a black background [11].

Haemolytic activity

The haemolytic activity of the *E.coli and Pseudomonas aeruginosa* was determined by zone of haemolysis around the colonies on blood agar plates containing 5 % (v/v) human blood, after 24 hours of incubation at 37^{o} C was considered as positive [12].

Beta lactamase production

Test organism was spot inoculated on to Mueller Hinton agar, penicillin and 1% starch agar medium and incubated overnight at 37^{o}C. After incubation, the plates were flooded with freshly prepared phosphate buffered saline

containing potassium iodide. The presence of clear colorless zones around the bacterial growth indicated the lactamase production. β-lactamase converts penicillin to penicilloic acid, which reduces iodine to iodide monitored via decolourisation of the starch iodine complex. All the bacterial isolates were tested for the production of β-lactamases [13].

Determination of virulence factors by genotypic methods

Isolation of genomic DNA

The genomic DNA was isolated from 1.5 ml of overnight culture in 2 ml micro centrifuge tubes. The tubes were centrifuged at 8000 rpm for 5 minutes. Then supernatant was discarded and pellet was collected. The pellet was suspended in 200 µl of 1X TE buffer + 100 µl of 10% SDS and mixed by vortexing and kept in water bath at 60°C for 20 minutes. Then added with 300 µl of phenol: chloroform: isoamyl alcohol mixture (24:25:1) to extract the DNA and mixed completely by vortexing and centrifuged at 10,000 rpm for 10 minutes to separate the phases. The aqueous phase containing the DNA was carefully removed and transferred to new tubes. Equal volume of 100% isopropanol was added to the tubes containing the aqueous phase. It was mixed by inverting the tubes 3 to 4 times. The tubes were then centrifuged at 10,000 rpm for 10 minutes to pellet the DNA. The supernatant was discarded and the pellet was collected. To the pellet, 200 µl of 70% ethanol was added and centrifuged at 10,000 rpm for 10 minutes. Then ethanol was decanted completely and the pellet was air-dried to give purified DNA. Re-suspended the dried DNA pellet in 20 µl of TE buffer and dissolved by tapping. DNA solutions were stored at 4°C for further work. The presence of DNA was confirmed by agarose gel electrophoresis [14].

Determination of *E.coli* virulence factor genes by using multiplex PCR

All the available partial and full-length gene sequences of resistance gene were determined according to Monique Ribeiro (2008) protocol with some modification. The primers was obtained from Sigma, India and used in the PCR comprised Primer CNF F 5' GAA CTT ATT AAG GAT AGT '3, and CNF R 5' CAT TAT TTA TAA CGC TG '3, PAP F 5' GCA ACA GCA ACG CTG GTT GCA TCA T '3 and PAP R 5' AGA GAG AGC CAC TCT TAT ACG GAC A'3, FIM F 5' TGC AGA ACG GAT AAG CCG TGG '3 and FIM R 5' GCA GTC ACC TGC CCT CCG GTA '3. The PCR mix were prepare in thin walled PCR tube in a sterile laminar flow hood, add the following reagents as follows.

Each PCR reaction mixture (20 µl) contained 2 µl of template DNA (Genomic DNA), 2 µl of 10 X PCR buffer, 0.5 µl of (0.5 µM) each of the primers, 1 µl of 0.2 mM of each deoxynucleotide triphosphate (dNTP'S) and 1 µl of Taq DNA polymerase (Con. 5U/ µl) and 11.0 µl of molecular grade water.

A brief spin was given to settle down the materials then tubes were kept in theromocycler (Genei). After initial denaturation at 94°C for 2 min, the samples were subjected to 30 cycles of denaturation at 94°C for 1 min, annealing at 62.5°C for 1 min and extension at 72°C for 1min. A final extension was performed at 72°C for 5 min. Following PCR, aliquots (20 µl) of the reaction mixtures were analyzed by electrophoresis on a 1.5% Agarose gel, containing ethidium bromide (0.2 mg/ml), in the presence of an appropriate DNA molecular weight marker. Then observe the amplification bands under UV transilluminater and detection of resistance gene with the use of marker [15].

Determination of *P.aeruginosa* virulence factor gene by using multiplex PCR

All the available partial and full-length gene sequences of resistance gene were determined according to Wozniak (1994) protocol with some modification. The primers was obtained from Sigma, India and used in the PCR comprised Primer ***alg*D1** 5'-AAG GCG GAA ATG CCA TCT CC-3' and ***alg*D2** 5'-AGG GAA GTT CCG GCC GTT TG-3'. The PCR mix was prepared in thin walled PCR tube in a sterile laminar flow hood and added the following reagents.

PCR reaction mixture (20 µl) contained 1µl of template DNA (Genomic DNA), 2 µl of 10 X PCR buffer, 0.3µl of (0.3 µM) each of the primers, 1 µl of 0.2 mM of each deoxynucleotide triphosphate (dNTP'S) and0.5 µl of Taq DNA polymerase (Con. 5U/ µl) and 14.9 µl of molecular grade water.

A brief spin was given to settle down the materials than tubes were kept in theromocycler (Genei). After initial denaturation at 94°C for 5 min, the samples were subjected to 30 cycles of denaturation at 94°C for 45 sec, annealing at 55.5°C for 30 sec and extension at 72°C for 1min. A final extension was performed at 72°C for 5 min. Following PCR, aliquots (20 µl) of the reaction mixtures were analyzed by electrophoresis on a 1.5% agarose gel, containing ethidium bromide (0.2 mg/ml), in the presence of an appropriate DNA molecular weight marker. Then

observe the amplification bands under UV transilluminater and detection of resistance gene with the use of marker [16].

Screening of ESBL pathogens against actinomycete extracts

About 6 actinomycetes isolates were obtained from actinomycetes research laboratory from Periyar University, Salem. Antibacterial activities of the extracts were tested against extended spectrum beta lactamase resistant pathogens by agar plug method. Agar plug were removed about 5mm diameter from 8 days grown cultures of the actinobacteria from ISP2 agar medium and then placed onto the nutrient agar plate which was previously swabbed with the test ESBL bacterial pathogens. All the plates were incubated at 37^{o} C for 24 hours and zone of inhibition was measured [17].

RESULTS

From the 40 samples (urine-25 and pus-15) 28 bacterial isolates were obtained. They are further confirmed as *Escherichia coli* (45%), *Klebsiella pneumoniae* (18%), *Staphylococcus aureus* (40%) and *Pseudomonas aeruginosa* (42%) by standard confirmatory tests. All the isolates subjected to antibiotic sensitivity test against third generation antibiotics (Table-1).

Table-1: Antibiotic sensitivity pattern of isolated strains

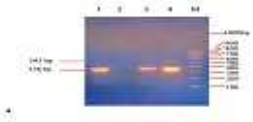
S.No	Isolates	Isolates name	ceftazidime	cephotaxime	cephadroxil	ciprofloxacin	cefoperazone
1)	*S.aureus*	S1	16mm	19mm	23mm	R	R
		S2	12mm	24mm	17mm	R	6mm
		S3	9mm	15mm	9mm	R	8mm
		S4	9mm	21mm	16mm	12mm	10mm
		S5	14mm	23mm	R	17mm	22mm
		S6	13mm	12mm	23mm	15mm	8mm
2)	*K.pneumoniae*	S7	14mm	9mm	12mm	17mm	R
		S8	R	11mm	19mm	13mm	R
		S9	15mm	9mm	8mm	11mm	8mm
		S10	16mm	12mm	9mm	8mm	R
3)	*E.coli*	S11	R	R	R	R	R
		S12	R	R	R	R	R
		S13	8mm	20mm	14mm	19mm	15mm
		S14	10mm	R	R	12mm	9mm
		S15	20mm	15mm	11mm	12mm	R
		S16	12mm	28mm	13mm	R	R
		S17	R	R	R	R	R
		S18	22mm	R	12mm	9mm	R
		S19	R	R	R	R	R
		S20	12mm	9mm	14mm	8mm	R
4)	*P.aeruginosa*	S21	9mm	15mm	14mm	8mm	R
		S22	12mm	9mm	17mm	11mm	R
		S23	13mm	16mm	8mm	17mm	8mm
		S24	R	R	R	R	R
		S25	11mm	15mm	10mm	8mm	R
		S26	22mm	10mm	6mm	13mm	7mm
		S27	20mm	14mm	17mm	R	R
		S28	11mm	15mm	12mm	18mm	R

Five strains (*E.coli*-S11, S12, S17, S19 and *P.aeruginosa*-S24) (18%) of bacteria were found to be β-lactamase producers. *Staphylococcus aureus* (S1-S6) and *Klebsiella pneumoniae* (S7-S10) are not producing ESBL. The five ESBL producing strain was further phenotypically and genotypically characterized for virulence of the strains. Of all 4 isolates of *E.coli* (S11, S12, S17, S19), 3 isolates were strongly positive (S11, S17 and S19) and strain S12 showed moderate positive for slime production. The strain S24 also showed strong positive. In case of haemolysis only two *E.coli* isolates (S11 and S12) exhibited lysis but *P.aeruginosa* (S24) does not produce haemolysis. In Beta lactamase assay of all resistance isolates showed positive result in starch medium. The result was observed after adding of 1% iodine solution to the plates. The clear halo zone was observed around the colonies that indicated as positive result (Table-2).

Table-2: Slime, Haemolytic and Beta lactamase production of isolated strains

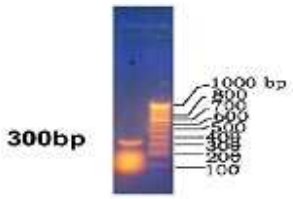
S.No	Isolates name	Slime production				Haemolytic	Beta lactamase
		Strong	Moderate	Week	Negative		
1.	*E.coli* (S11)	+	-	-	-	+	+
2.	*E.coli* (S12)	-	+	-	-	+	+
3.	*E.coli* (S17)	+	-	-	-	-	+
4.	*E.coli* (S19)	+	-	-	-	-	+
5.	*P.aeruginosa* (S24)	+	-	-	-	-	+

Figure 1: Virulence genes identification for genotypic method



Lane 1 to 4 – *E.coli* isolates, Lane M – 100 bp DNA marker. It shows the virulence genes amplication for *E.coli* by multiplex PCR method. Virulence genes used are *fim*, *pap*, *cnf*. In this study *pap* and *cnf* amplified and *fim* gene not produced. 3 isolates amplified *pap* gene (Lane 1, 3 and 4 which of 336 bp). Only 1 isolate amplified *cnf* gene (Lane 4 which of 543 bp).

Figure 2: Virulence genes identification for genotypic method

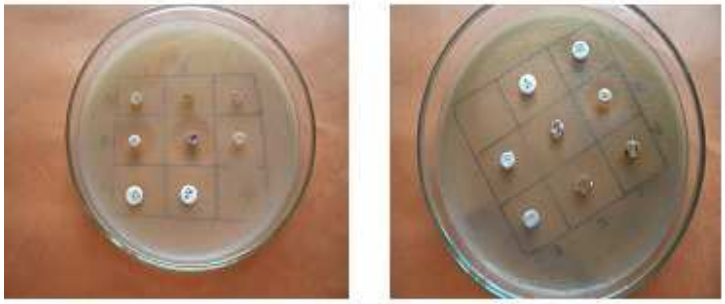


Lane 1 – *P.aeruginosa* isolate, Lane M- 100 bp DNA marker. Only one virulence gene used for *P.aeruginosa* is *alg* D, amplified up to 300 bp.

In this current study 3 types of primers were used for amplification of virulence genes such as *pap*, *cnf* and *fim* for *E.coli*. Among them 2 virulence genes were amplified namely *pap* and *cnf*. In this investigation 75% of *pap*

virulence genes were amplified and 25 % were *cnf* but *fim* gene was not amplified by all the four *E.coli* resistant isolates (Figure-1). For *P.aeruginosa* only one primer were used for amplification of virulence gene namely *alg* D. Virulence genes were amplified according to the base pairs. In this investigation 100% of *alg* D virulence gene was amplified (Figure-2) . In the antagonistic activity study all five isolates showed resistant to the extracts of Pm33, Pm30 and Pm16. R2 showed inhibitory activity against only one isolates namely S12 and other isolates showed resistance to the R2 extracts. *Streptomyces* sp. of Y19 and PE7 extracts showed highest antagonistic activity against 4 isolates namely (S11, S17, S19 and S24) and no activity for S12 isolates. Y19 and PE7 extract contains the metabolites have a prominent capacity to control the ESBL pathogens (Figure-3).

Figure 3: Effect of Actinomycetes extracts against ESBL producers



E.coli S11 *P.aeruginosa* S24

Plates shows that ESBL resistant and virulence factors of *E.coli* S11 and *P.aeruginosa* S24 against Actinomycetes extracts

In the antagonistic activity study all five isolates showed resistant to the extracts of P33, P30 and P16. P6 showed inhibitory activity against only one isolates namely S12 and other isolates showed resistance to the P6 extracts. *Streptomyces* sp. of Y1 and P7 extracts showed highest antagonistic activity against 4 isolates namely (S11, S17, S19 and S24) and no activity for S12 isolates. Y1 and P7 extract contains the metabolites have a prominent capacity to control the ESBL pathogens (Figure-3).

DISCUSSION

Bacterial resistance to β-lactam antibiotics has significantly increased in recent years. This increase has been attributed to the spread of plasmid-mediated extended spectrum β-lactamases (ESBLs). ESBLs occur predominantly in the family of *Enterobacteriaceae*. *Klebsiella pneumoniae* and *E.coli* are the main species in which ESBL enzymes have been most commonly reported worldwide. Urinary tract infection was one of the commonest bacterial infections. *Enterobacteriaceae* were the most frequent pathogens detected in UTI. In this study the organisms like *E.coli* (45%), *P.aeruginosa* (42%), *Staphylococcus aureus* (40%) and *K.pneumoniae* (18%), isolated from patients suffering from UTI and wound infections. Similarly, Rubeena Hafeez *et al*., 2009 isolated the organisms like *E.coli* (44.8%), followed by *K.pneumoniae* (38.6%), *P.mirabilis* (31.6%) and *A.baumanni* (71%) from clinical samples [18]. Increase in antibiotic resistance among bacterial pathogens is major problems in medicine and science today. Among the ESBL pathogens *E.coli* is much high in human UTI. It is also evident that *E.coli* possesses potential virulence factors selectively attached to the mucosa of uro-epithelium, colonized and persist in urinary tract [19]. Similarly [20] obtained the antibiotic resistance isolates showed resistance to many of third generation antibiotics. Previously [21] evaluated the pattern and prevenlance of unrinary tract infections mainly comprised of *Klebsiella pneumoniae* and *Proteus* sp., *Pseudomonas aeruginosa*, *Enterococcus* sp., and *Streptococcus agalactiae* in salem. Among the all pathogens the amount of gram negative strains particularly E.coli is the major causative agent for Urinary tract infection.

All ESBL resistance isolates were subjected to virulence characterization. In bacteria, virulence factors elucidate the pathogenicity and attached to the host cells to develop the diseases. Some of the virulence factors are slime production, haemolysis and β-lactamase enzyme. In this study slime production by the strains was significant and showed the slime production plays a significant role in the determining the binding and intracellular penetration of polysaccharide that vary in charge and hydrophobicity and generate the reactive oxygen inside the cell lead to the death [22]. It is also reported that slime production makes the strain as virulent and allow binding to the protein coat of the foreign bodies and initiate the biofilm formation [23]. The importance of the role played by slime was further increased by its frequent association to reduced antibiotic susceptibility [24]. The difficulty in eradicating a chronic infection associated with slime formation has been reported and slime-producing bacteria have been shown to resist higher antibiotic concentrations than non-slime producing bacteria [25].

Further the hemolysis of the strain confirms its pathogenicity phenotypically. [26] also stated that production and presence of haemolysis in *Aeromonas hydrophila* leads to the pathogenicity. Prevalence of ESBL production occurrence was higher in hospitalized patients. Then β-lactamase enzyme production was confirmed in Mueller-Hinton agar by using the antibiotics. This enzyme produced from the microorganisms that cleave the β-lactam ring present in the antibiotic and inactivate the antibiotics. Along with the virulence factors the extracellular enzymes produced by the pathogenic strains aid their resistance to host defenses as well as to many antibiotics.

In the present study, it is evident that apart from enzyme virulence factors, genes are also responsible for emerging diseases. The results showed high frequency in *pap* genes (75%) compared to other genes and this clearly indicates that *pap* gene is responsible for *E.coli*. Similarly Monique Ribeiro amplified *fim, pap, cnf* genes used for *E.coli* and obtained the results in the percentage of *fim* (97.5%), *pap* (32.7%), *cnf* (18.5%). For *Pseudomonas alg* D gene used, it encodes GDP-mannose dehydrogenase, an enzymes converts GDP-mannose to GDP-mannuronic acid. Over production of alginate leads to morbidity and mortality of individuals associated with cystic fibrosis. In this study 100% *alg* D gene amplified, for Wound infected patients. Actinomycetes have been recognized as a source of several secondary metabolites like antibiotics and lytic enzymes.

The *Streptomyces* sp. has shown to have characteristics bioactive compounds which make them useful as antagonistic agents against pathogens [27]. This study clearly showed that actinomycetes are having potential ability to control the virulence pathogens. Among the six actinomycetes extracts tested P7, Y1 showed highest inhibitory activity against virulent pathogens. Similar to the current study [28] reported the actinobacterial activity against ESBL pathogens. The previous report of [29] was also evidenced the antimicrobial activity of *Streptomyces* sp. against antibiotic resistance isolates. Recently, upcoming of new type of metallo β-lactamase producing strains are reported in Bangalore hospitals [30]. This also evidenced that ESBL producing pathogens spreading to the towns apart from cities. This supports to the present study and also reveals that prevalence of ESBL producing strains in Salem town. Actinomycetes particularly *Streptomyces* sp., isolated from extreme environments like marine, desert, forest and endophytic actinomycetes are rich source for new active drug target in future was evidenced by [31,32, 33].

CONCLUSION

The present study clearly states that the *Streptomyces* sp. Y1 and P7 showed highest activity against gram negative isolates of *E.coli* and *Pseudomonas aeruginosa*. These findings indicated that produced bioactive compounds may be the best antibiotics for controlling ESBLs. Future research in this area to find out the bioactive metabolite against resistant pathogens is essential. So this type of study from actinomycetes from unexplored extreme environment is economically viable and new insight into the development of novel antibiotics to the welfare of human health by overwhelming the problems due to multidrug resistance.

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REFERENCES

[1]Paterson DL, Hujer KM, Hujer AM, Yeiser B, Bonomo MD, Rice LB, *Antimicrob Agents Chemother*, **2003**, 47, 3554-60.

[2]Page ML, *ASM News*, **2002**, 68, 217-21.
[3]Kenneth T, *Textbook of Bacteriology*, **2008**, 304, 14.
[4]Fred C, Jasmine Mohammed M, Timothy S, Dembek F, *J Clin Microbial*, **1999**, 37, 4065-4070.
[5]Shiju MP, Yashavanth R, Narendra N, *Journal of Clinical and Diagnostic Research*, **2010**, 4, 2442-2445.
[6]Bachmann SL, McCarthy AJ, *Applied Environ Microbiol*, **1991**, 57, 2121-2130.
[7]Miyadoh S, *Actinomycetologica*, **1993**, 7, 100-106.
[8]Cheesbrough M, Medical Laboratory manual for tropical countries, Microbiology. Cambridge, Great Britain, **1989**, 2, 248-263.
[9]Bauer AW, Kirby WM, Sherris JC, Turck M, *AMJ Clin Pathol*, **1966**, 45, 493-496.
[10] National committee for Clinical Laboratory standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard. 5th ed. NCCLS document M7-A5. Wayne, PA: NCCLS, **2000**.
[11] Freeman DJ, Falkiner FR, Keane CT, *J Clin Pathol*, **1989**, 42, 872-874.
[12] Brenden R, Janda JM, *J Med Microbiol*, **1987**, 24, 247-251.
[13] Lateef A, Oloke JK, Gueguim Kana EB, *African Journal of Biotechnology*, **2004**, 3, 334-338.
[14] Un-Ho Jin, Sung-Hak Cho, Min-Gon Kim, Sang-Do Ha Keun-Sung Kim, Kyu-Ho Lee, Kwang-Yup Kim, Duck Hwa Chung, Young Choon Lee, Cheorl-Ho Kim, *The Journal of Microbiology*, **2004**, 42, 216-222.
[15] Ribeiro M, Yano M, Silva D, *Rev Inst Med Trop*, **2008**, 50, 255-260.
[16] Wozniak J, *Journal of Bacteriology*, **1994**, 176, 5068-5078.
[17] Balagurunathan R, Mohanraj D, Bharathi S, Radhakrishnan M, *J Chem Pharm Res*, **2011**, 3, 439-446.
[18] Hafeez R, Aslam M, Farzana M, Javaid M, Ajmal AN, *Biomedica*, **2009**, 25, 112-115.
[19] Alo MN, Anyim C, JC Igwe, Elom M, *Advances in Applied Science Research.*, **2012**, 3, 821-825.
[20] Abdulrahaman AK, Kumar A, *Journal of Saudi Medical*, **2005**, 25, 239-242.
[21] Shanthi J, Kayathri S, *Advances in Applied Science Research*, **2012**, 3, 3410-3414.
[22] Gad F, Zahra T, Hasan T, Hamblin MR, *Antimicrobial agents and Chemotheraphy*, **2004**, 48, 6.
[23] Goldmann DA, Pier GB, *Clin Microbiol Rev*, **1993**, 6, 176–192.
[24] Kloss WE, Bannerman TL, *Clin Microbiol Rev*, **1994**, 7, 117-140.
[25] Gristina AG, Hobgood CD, Webb LX, Myrvik QN, *Biomaterials*, **1987**, 8, 423-426.
[26] Vijay Singh, Chaudhary DK, Indra Mani, Somvanshi P, Rathore G, *African Journal of Microbiology*, **2010**, 4, 952-957.
[27] Kariminik A, Baniasadi F, *World Applied Sciences Journal*, **2010**, 8, 828-832.
[28] Hemachandran K, Bharathi S, Radhakrishnan M, Balagurunathan R, *Journal of Applied Pharmaceutical Science*, **2011**, 1, 210-213.
[29] Ceylan O, Okmen G, Ugur A, *Eur Asia J BioSci*, **2008**, 2, 73-82.
[30] John S, Balagurunathan R, *Indian Journal of Medical Microbiology*, **2011**, 29, 302-304.
[31] Srividya S, Adarshana Thapa, Deepika V Bhat, Kajingailu Golmei, Nilanjan Dey, *European Journal of Experimental Biology*, **2012**, 2, 163-173.
[32] SR Krishna, SR Sathish Kumar, L Meenambekha, M Madhusudhan, *Advances in Applied Science Research*, **2011**, 2, 431-439.
[33] Kumud Chandra Kandpal, DA Jain, Umesh Kumar, Rashmi Tripathi, T Siva Kumar, *European Journal of Experimental Biology*, **2012**, 2, 1733-1737.