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Isolation and Identification of Antibiotic Resistant and Metal Tolerant Bacteria from Soil Samples of Akola district of Maharashtra, India.

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Abstract:
This study was conducted to investigate the antibiotic and heavy metal tolerance profile of bacterial pathogens isolated from farmland in Akola. Different soil samples were collected and processed following standard procedures. Each sample was then inoculated onto culture media. Identification, Antibiotic susceptibility testing of each isolate was carried out by the Kirby-Bauer disc diffusion method. The heavy metal susceptibility testing of bacterial isolate were determined by the Agar-well diffusion method. The isolates were identified as Pseudomonas aeruginosa, Escherichia coli, Staphylococcus epidermidis, Azatobacter spp. and Streptococcus pyogenes. The present study reveals that the P. aeruginosa showed a high frequency of multiple resistance to antibiotics. The isolates were highly resistant to low concentration of heavy metals such as Copper, Zinc and Lead. Bacterial resistance to both antibiotic and heavy metals can readily be isolated from the natural environment.

Keywords*: Soil micro-organisms, Antibiotic resistance, Heavy metal tolerance, Agar-well diffusion method, Disc diffusion method.*

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I. Introduction:

Soil contamination is caused by the presence of xenobiotic chemicals or other alterations in the natural soil environment. Contamination is typically caused by the accumulation of heavy metals and metalloids through emission from the rapidly expanding industrial areas, disposal of high metal wastes, leaded gasoline and paints, etc. Application of fertilizers, sewage sludge, pesticides, waste water irrigation, coal combustion residues, spillage of petrochemicals and the atmospheric deposition from smelting also possess a threat to environmental sustainability (Zhang M-K, Liu Z-Y, Wang H, 2010).

Metals play an essential role in the metabolic processes of the biota. Some of the heavy metals are essential and are required by the organisms as micronutrients (Cobalt, Nickel, Iron, Zinc, etc) and are known as "trace elements" (Bruins, M.R., SS. Kapil and F.W. Oehme, 2000). On the other hand, there are metals such as Pb, Cd, Hg, etc. which have no biological role, instead are toxic and detrimental even at very low concentrations. However at higher concentrations, metals, both essential as well as non-essential, are toxic.

However, elevated levels of heavy metals decrease soil microbial action and crop production (Mclaughlin, M.J., D.R. Parke and J.M. Clarke, 1999). These heavy metals influence the microbial population by affecting their growth, morphology, biochemical activities and ultimately resulting in decreased biomass and diversity (Roane, T.M. and I.L. Pepper, 2000). Furthermore, increasing concentration of metals beyond tolerance level have forced these organisms to adapt to various biological mechanisms such as efflux system, complexation.

Antibiotic resistance has become a major health hazard due to its use and misuse. Antibiotic resistant bacteria are now found in large numbers in virtually every ecosystem on earth. Resistance to antibiotics is acquired by a change in the genetic makeup of a bacterium which can occur by either a genetic mutations or by transfer of antibiotic resistance genes between bacteria in the environment disinfectants (Summers, A.O., 2000). Antibiotic resistance and metal tolerance by bacteria species are common phenomenon because metal exposure by bacteria selects for bacteria resistant to antibiotics (Summers, A.O., 2002).

Therefore, in the present study an attempt was made to determine antibiotic resistant and heavy metal tolerance of bacterial isolates from soils that are not affected by clinical waste, but subjected to probable heavy metal contamination.

II. Materials and Methods:

Collection of soil sample:

Fifty two soil samples were collected from four different locations of Akola district of Maharashtra, India. The sampling sites were agricultural land, public grounds, uncultivable lands and industrial area. Soil samples from a depth of 15 to 20 cm from the surface were collected after removing the top layer. For each of the sampling sites, sub-samples of soil were collected from different locations, pooled together and homogenized so as to obtain the presentative sample. The samples were collected using a spade that is thoroughly cleaned and disinfected between sampling so as to prevent cross contamination. The samples collected were transported to the Microbiology laboratory of Shri Shivaji College of Arts, Commerce and Science, Akola for analysis (Stanley Chukwudozie Onuoha, *et al*., 2016).

Isolation of bacteria:

A serial dilution of samples (1gm in the case of fresh soil) was made using steriled distilled water until a dilution of 10^{-5} was obtained. The surface of molted Nutrient agar medium was flooded with 1 ml of diluents each on a separate prepared Nutrient agar plates. The plates were incubated at 37ºC for 18 to 24 hours. Physical colonies were counted and growth was subcultured to obtain a pure culture for further identification and characterization (Silpa Sivan, Akhil Venu, *et al*., 2015).

Identification and Characterization of the Isolates:

After 18 to 24 hours of incubation, isolates on the media (pure colonies) obtained were further characterized and identified using standard microbiological and biochemical techniques such as morphological test, Gram staining, Indole test, MR test, VP test, Citrate utilization test, sugar fermentation, Oxidase test, Catalase test, Gelatinase test, Urease test, Caseinase test and Dnase test (S. Silambarasan and A. Jayanthi, 2010).

Antibiotic Susceptibility Test:

The antibiotic susceptibility pattern of the bacteria isolates were determined by the modified Kirby and Bauer disc diffusion susceptibility test method as recommended by NCCLS (Now CLSI). An overnight culture of the bacteria grown on nutrient broth was used as Inoculum. The inoculums were aseptically swabbed on the surface of nutrient agar plates using steriled swab sticks. Commercially available antibiotic disc impregnated with the following: Gentamycin (10 µg), Erythromycin (15 µg), Chloramphenicol (30 µg), Amoxyclav (30 µg), Ampicillin (10 µg), Tetracycline (30 µg), Ciprofloxacin (5 µg) were aseptically placed on the nutrient agar plates. The plates were incubated at 37ºC for 18 to 24 hours and the inhibition zone diameter produced by the antibiotic disc was measured using a meter rule and was recorded (Cabral L, *et al*., 2016).

Heavy Metal Tolerant Test:

The heavy metal susceptibility testing of bacteria isolates were determined by the Agar-well diffusion method as recommended by CLSI. To examine the ability of the isolates to resist heavy metals, cells of overnight grown cultures were inoculated on Muller Hinton Agar plates. Using steriled well borer, the wells were made on the surface of Muller Hinton Agar plate. The heavy metal solutions (copper in copper sulphate, zinc in zinc sulphate and lead in lead acetate) of different concentrations i.e.10 mM, 50 mM, 100 mM, 250 mM, 500 mM were prepared. Then 100 µl of the standard metal salt solution of different concentrations was poured into the well and was allowed to diffuse evenly across the well. The plates were incubated at 37ºC for 24 hours. After incubation, the inhibition zone diameter was measured using a meter rule and was recorded (Smriti Singh, *et al*., 2014).

III. Results and Discussion:

Sampling:

To study the isolation and identification of antibiotic resistant and metal tolerant bacteria from soil samples. Fifty two soil samples were collected from the Agricultural and Industrial areas of Akola.

Isolation of bacterial species:

The five different bacterial species were isolated from soil samples. The isolated bacterial species were identified as *P. aeruginosa, E. coli, S. epidermidis, Azatobacter spp*. and *S. pyogenes* on the basis of morphological and biochemical characteristics.

Table 1: Colony characters of isolated bacteria on Nutrient agar media after 24 hours at 37º C were observed as follows-

Colony characters	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
Size	Large	Large	Small	Large	Small
Shape	Round	Circular	Round	Flat	Dome-shaped
Margin	Entire	Entire	Entire	Undulate	Clear
Elevation	Umbonate	Convex	Raised	Convex	Convex

Colour	Green colony	White colony	Cream	Colourless	White colony
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Texture	Smooth	Smooth	Smooth	Smooth	Smooth
Motility	Motile	Motile	Non- motile	Motile	Non-motile
Gram character	Gram –ve rods	Gram –ve rods	Gram +ve cocci	Gram –ve rods	Gram +ve cocci in chains
Confirmed Isolate	*Pseudomonas aeruginosa*	*Escherichia coli*	*Staphylococcus epidermidis*	*Azatobacter spp.*	*Streptococcus pyogenes*

(where +ve = positive, -ve = negative)

Table 2 : Biochemical characteristics of isolated bacteria

Sr. no.	Biochemical test	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
1	Indole test	-ve	+ve	-ve	+ve	-ve
2	Methyl-Red test	-ve	+ve	-ve	+ve	+ve
3	Voges-Proskauer test	-ve	-ve	+ve	-ve	-ve
4	Citrate utilization test	+ve	-ve	-ve	+ve	-ve
5	Catalase test	+ve	+ve	+ve	+ve	-ve
6	Oxidase test	+ve	-ve	-ve	+ve	-ve
7	Urease test	-ve	-ve	+ve	+ve	-ve
8	Gelatin liquification test	+ve	-ve	-ve	-ve	-ve
9	Caseinase test	+ve	-ve	+ve	+ve	+ve
10	Dnase test	-ve	-ve	-ve	+ve	+ve
11	Sucrose	-ve	+ve	+ve	+ve	+ve
12	Lactose	-ve	+ve	+ve	+ve	+ve
13	Xylose	-ve	+ve	-ve	-ve	-ve
14	Mannitol	+ve	+ve	-ve	+ve	-ve
15	Confirmed isolate	*Pseudomonas aeruginosa*	*Escherichia coli*	*Staphylococcus epidermidis*	*Azatobacter spp.*	*Streptococcus pyogenes*

(where +ve = positive, -ve = negative)

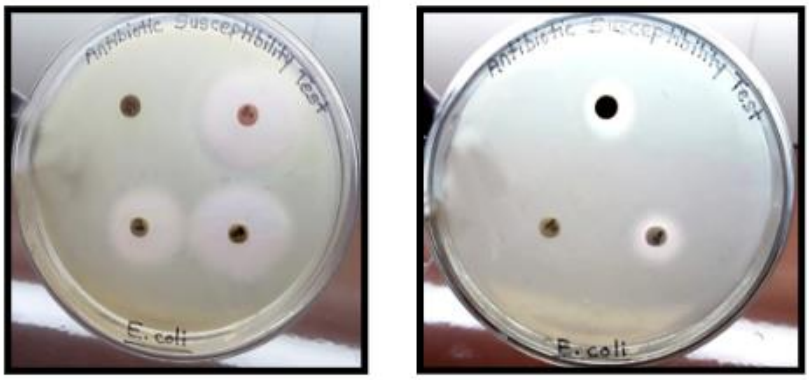
Antibiotic susceptibility test:

The present study reveals that the *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* showed a high frequency of multiple resistance to antibiotics.

The *P. aeruginosa* was resistant to Ampicillin, Amoxyclav and Tetracycline while *S. epidermidis* was resistant to Ampicillin, Amoxyclav, Tetracycline and Erythromycin. The *P. aeruginosa* was sensitive to Gentamycin, Erythromycin, Chloramphenicol and Ciprofloxacin. The *S. epidermidis* was found to be sensitive to Gentamycin, Chloramphenicol and Ciprofloxacin while *E. coli* and *Azatobacter spp*. were sensitive to Gentamycin, Erythromycin, Chloramphenicol, Tetracycline and Ciprofloxacin.

E. coli and *Azatobacter spp*. was found to be resistant to Ampicillin and Amoxyclav. The *S. pyogenes* was resistant to Ampicillin and Amoxyclav and sensitive to the rest.

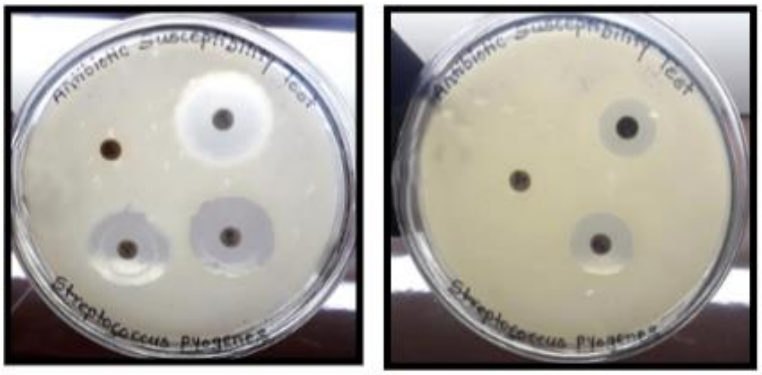
Antibiotic Susceptibility Test for *P. aeruginosa*



Antibiotic Susceptibility Test for *E. coli*

Antibiotic Susceptibility Test for *S. epidermidis*

Antibiotic Susceptibility Test for *Azatobacter spp*

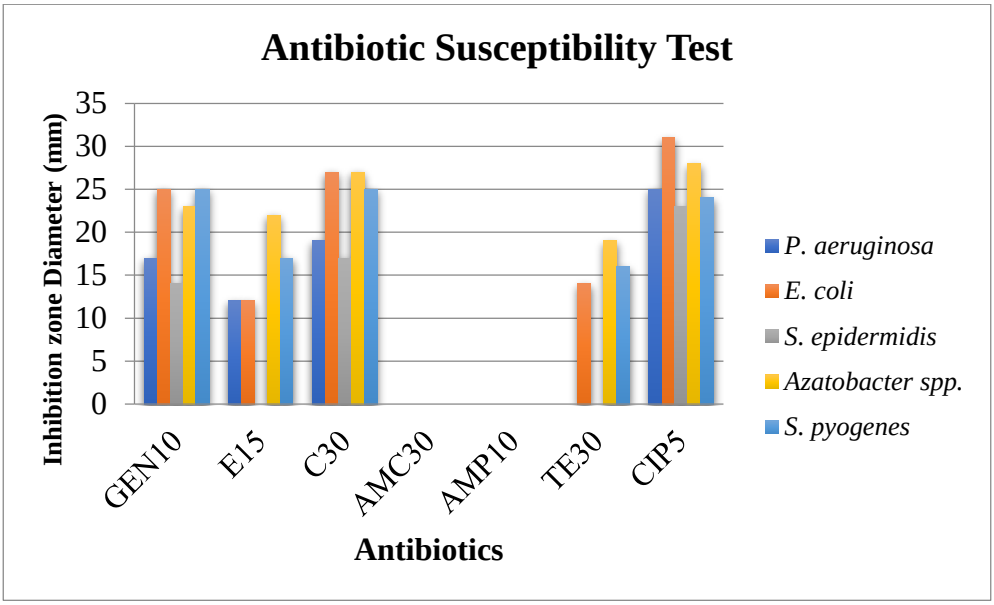


Antibiotic Susceptibility Test for *S. pyogenes*

Table 3: Antibiotic sensitivity and resistance pattern of isolated bacteria (expressed in mm)

Name of antibiotics	*P. aeruginosa*	*E. coli*	*S. epidermidis*	*Azatobacter spp.*	*S. pyogenes*
Gentamycin (GEN^{10})	17 mm	25 mm	14 mm	23 mm	25 mm
Erythromycin (E^{15})	12 mm	12 mm	NI	22 mm	17 mm
Chloramphenicol (C^{30})	19 mm	27 mm	17 mm	27 mm	25 mm
Amoxyclav (AMC^{30})	NI	NI	NI	NI	NI
Ampicillin (AMP^{10})	NI	NI	NI	NI	NI
Tetracycline (TE^{30})	NI	14 mm	NI	19 mm	16 mm
Ciprofloxacin (CIP^{5})	25 mm	31 mm	23 mm	28 mm	24 mm

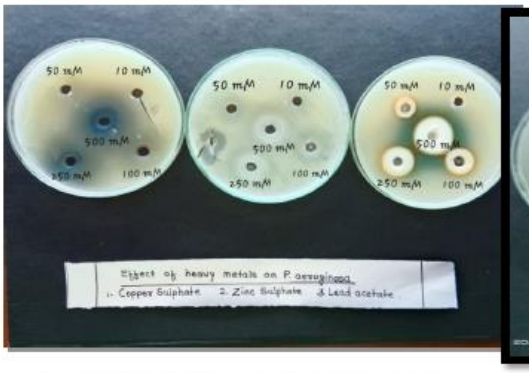
(NI = No inhibition zone ; Diameter of disc = 6 mm)



Heavy metal tolerance assay:

The present study reveals that *P. aeruginosa*, *E. coli* and *Azatobacter spp*. were found to tolerate copper even upto 100 mM, whereas *S. pyogenes* could tolerate copper only upto 50 mM and *S. epidermidis* could tolerate copper only upto 10 mM. *P. aeruginosa* and *S. epidermidis* were sensitive to zinc even at its low concentration. Whereas, *Azatobacter spp* and *S. pyogenes* were found to tolerate zinc only upto 10 mM and *E. coli* could tolerate zinc upto 50 mM. All bacterial isolates can tolerate lead at its low concentration and sensitive to lead at its higher concentration.

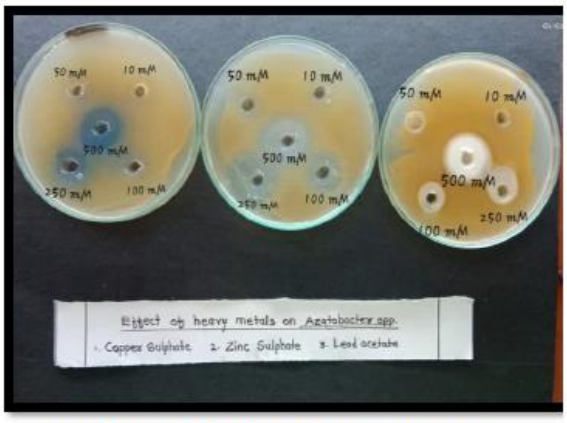
The results showed that the *Escherichia coli* is the most commonly reported bacteria in soil, and possess much higher tolerance to heavy metals such as copper, lead and zinc. The present study reveals that *P. aeruginosa* and *Azatobacter spp*. are the most commonly reported bacteria in soil and posses much higher tolerance to copper only.



Heavy Metal Susceptibility Test for *P. aeruginosa*

Heavy Metal Susceptibility Test for *E. coli*

Heavy Metal Susceptibility Test for *S. epidermidis*



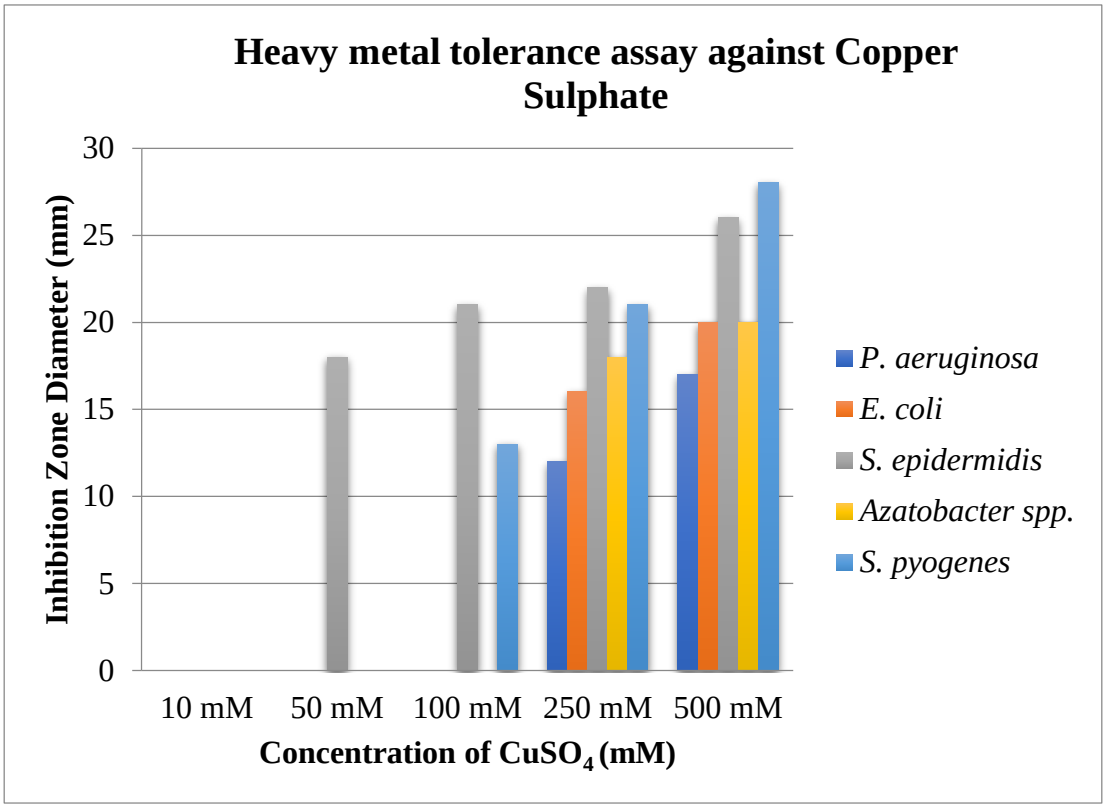
Heavy Metal Susceptibility Test for *Azatobacter spp.*

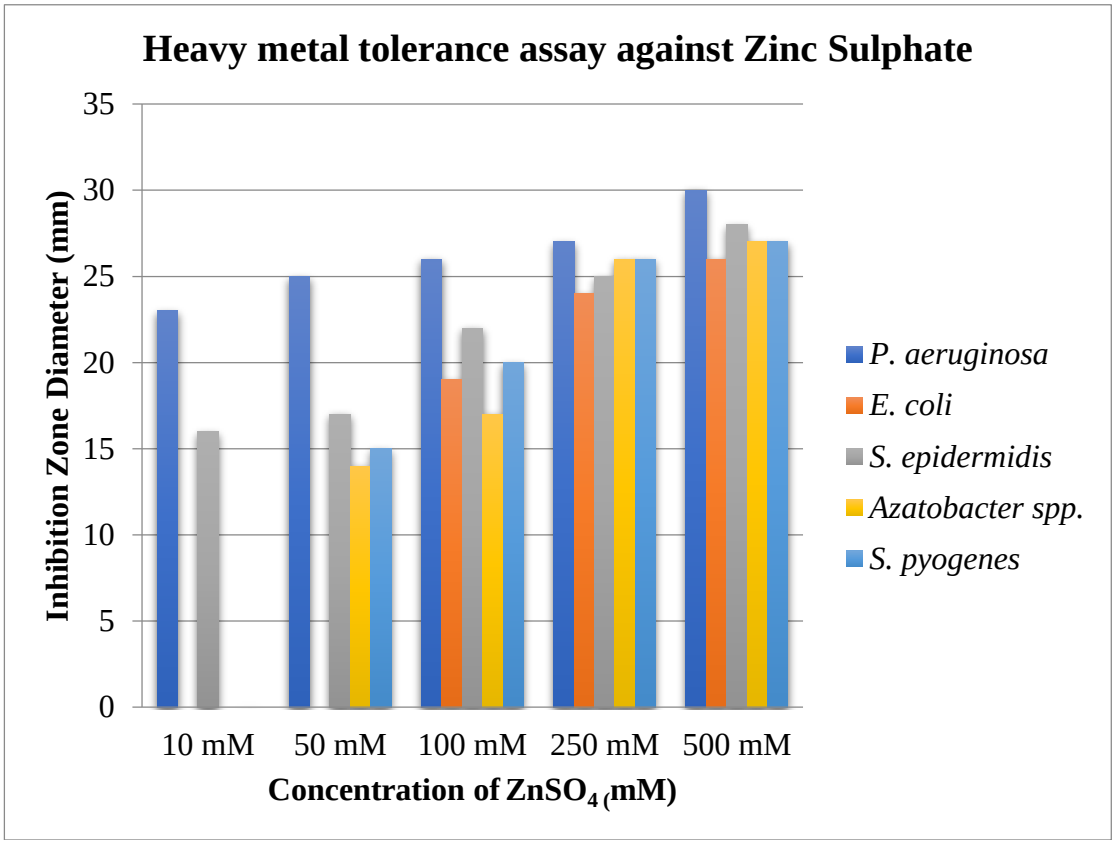
Heavy Metal Suceptibility Test for *S. pyogenes*

Table 4: Heavy metal tolerance pattern of isolated bacteria (expressed in mm)

Zone of inhibition (in mm) on agar plates with different metal concentrations															
Isolates	**Conc. Of CuSO4 (in mM)**					**Conc. Of ZnSO4 (in mM)**					**Conc. Of lead acetate (in mM)**				
	10	**50**	**100**	**250**	**500**	**10**	**50**	**100**	**250**	**500**	**10**	**50**	**100**	**250**	**500**
P. aeruginosa	NI	NI	NI	12	17	23	25	26	27	30	NI	13	18	21	26
E. coli	NI	NI	NI	16	20	NI	NI	19	24	26	NI	NI	17	24	25
S. epidermidis	NI	18	21	22	26	16	17	22	25	28	NI	NI	17	22	25
Azatobacter spp.	NI	NI	NI	18	20	NI	14	17	26	27	NI	11	15	18	27
S. pyogenes	NI	NI	13	21	28	NI	15	20	26	27	NI	12	19	26	29

(NI = No inhibition zone)





IV. Discussion:

Even metals exert their toxic effects on microorganisms through various mechanism, and metal tolerant bacteria could survive in these habitats and could be isolated and selected for their potential application in the bioremediation of contaminated sites (Piotrowska-seget *et al*., 2005). In the present study, we have isolated five

bacterial isolates which could be resistant to copper, zinc and lead. The five heavy metal tolerant and antibiotic resistant bacteria isolated were *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus epidermidis, Azatobacter spp.* and *Streptococcus pyogenes.* These results are similar to the report of Kolawole and Obueh (2015) that isolated *Staphylococcus spp., E. coli, Klebsiella, Salmonella* and *Pseudomonas spp.* as heavy metal tolerant bacteria.

In the present study, we have isolated five bacterial isolates namely *P. aeruginosa, E. coli, S. epidermidis, Azatobacter spp.* and *S. pyogenes*. The antibiotic susceptibility pattern of the bacterial isolates were determined by the modified Kirby and Bauer disc diffusion susceptibility test method. It was found that all isolates were resistant to some antibiotics which mainly includes Ampicillin and Amoxyclav. But, the *P. aeruginosa* and *S. epidermidis* exert maximum resistance against antibiotics such as Amoxyclav, Ampicillin, Tetracycline and Erythromycin. These results are similar to the report of N.L.M. Budambula and D.M. Kinyua (2014) that isolated *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis, Citrobacter freundii, Serratia marscens* as multi-drug resistant bacteria. They found that these isolates were resistant to Augmentin, Cefuroxime, Ampicillin and Cotrimoxazole.

Antibiotic resistance and Metal tolerance were found in bacterial species isolated from contaminated industrial and agricultural soil. The isolates were identified as *P. aeruginosa, E. coli, S. epidermidis, Azatobacter spp. and S. pyogenes.* All bacterial isolates can tolerate lead and copper at its low concentration. The result were similar to the previous works reporting the presence of these bacterial isolates in metal contaminated environments. (S. Silambarasan and A. Jayanthi, 2010). They found that all these isolates were tolerant to lead, chromium, copper, nickel and manganese.

The inhibitory effects of higher concentrations of heavy metals to the isolates were most probably due to surface binding and disruption of membrane function (Clement, J.L. and P. Jarrett, 1994). For instance, the mechanism of resistance to lead might be due to an efflux by P-type ATPases and intracellular compounds complexation (Nies, D., 1999). Tolerance to Cu^{2+} is due to abililty of the isolates to accumulate Copper ions in its cell wall thus preventing its entry into the cell (Beveridge, T. J. and R. J. Doyle, 1989). However, at higher concentrations there is oxidation of lipid membranes, damage to nucleic acid (Lippert, B., 1992) and generation of free radicals from Hydrogen peroxide (Dameron, C.T. and M.D. Harrison, 1998).

Bacterial resistance to both antibiotics and heavy metals can readily be isolated from the natural environment, with greater abundance noted for Agricultural and Industrial sites of Akola. The presence of bacteria capable of tolerating heavy metals from contaminated soil was investigated. Bacteria that are capable of tolerating heavy metals were isolated in pure cultures, where five isolates were identified. The study demonstrates metal tolerance by *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus epidermidis, Azatobacter spp. and Streptococcus pyogenes. Pseudomonas aeruginosa* exert maximum tolerance against copper, and *E. coli* against zinc, lead and copper.

V. Conclusion:

Bacterial resistance to both antibiotics and heavy metals can readily be isolated from the natural environment, with greater abundance noted for Agricultural and Industrial sites of Akola.

In summary, the results presented in this work revealed that the five isolates, characterized with remarkable tolerance to heavy metals could be potential indicator of toxicity of heavy metals to other forms of life. The present study revealed that there is evidence of a relation between tolerance to heavy metals and antibiotic resistance. Thus, industrial contamination leads eventually to more resistant strains resulting in treating infections difficult causing a major global problem. Microbial interactions with metals may have several implications for the environment. Microbes may play a large role in the biogeochemical cycling of toxic heavy metals which can be used in cleaning up or remediating metal contaminated environments.

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References:

[1]. Silambarasan, S., & Jayanthi, A. (2010). A Study on Antibiotic Resistance and Metal Tolerance of Bacteria Isolated from Industrial Site. Nature, Environment and Pollution Technology, **9**(2), 261-266.

[2]. Sivan S., Venu A., and Joseph J. (2015). Isolation and Identification of Heavy Metals Tolerant Bacteria from Industrial and Agricultural Areas in Kerala. International Journal of Multidis Ciplinary Research. **1**(4). ISSN; 2395-6968.

[3]. Stanley Chukwudozie Onuoha, Collins Onyebuchi Okafor and Bruno C. Aduo (2016): Antibiotic and Heavy Metal Tolerance of Bacterial Pathogens Isolated from Agricultural soil.World Journal of Medical Sciences. **13**(4): 236-241. ISSN: 1817-3055.

[4]. Singh, S., Pandey, S., & Chaudhary, H. S. (2014). Actinomycetes: tolerance against heavy metals and antibiotics. Int J Bioassays, 3, 3376-3383.

[5]. Zhang, M. K., Liu, Z. Y., & Wang, H. (2010). Use of single extraction methods to predict bioavailability of heavy metals in polluted soils to rice. Communications in soil science

[6]. Bruins, M. R., Kapil, S., & Oehme, F. W. (2000). Microbial resistance to metals in the environment. Ecotoxicology and environmental safety, **45**(3), 198-207.and plant analysis, **41**(7), 820-831.
[7]. McLaughlin, M. J., Parker, D. R., & Clarke, J. M. (1999). Metals and micronutrients–food safety issues. Field crops research, 60(1-2), 143-163.
[8]. Roane, T. M., & Pepper, I. L. (1999). Microbial responses to environmentally toxic cadmium. Microbial ecology, 358-364.
[9]. Summers A.O., 2000. Generally overlooked fundamentals of bacterial genetics and. ecology. Clin Infect.Dis., 33: S85-S92.
[10]. Beveridge, T. J., & Doyle, R. J. (1989). Metal ions and bacteria. Wiley. Antibiotic-selective environments. Clin.Infect.Dis., 27: S5-S11.
[11]. Lippert, B. (1992). From cisplatin to artificial nucleases—the role of metal ion-nucleic acid interactions in biology. Biometals, 5, 195-208.
[12]. Dameron, C. T., & Harrison, M. D. (1998). Mechanisms for protection against copper toxicity. The American journal of clinical nutrition, **67**(5), 1091S-1097S.
[13]. Kolawole, S. E., & Obueh, H. O. (2015). Evaluation of the minerals, heavy metals and microbial compositions of drinking water from different sources in Utagba-Uno, Nigeria. J. Health Environ. Sci, 2, 6-10.
[14]. Clement, J.L. and P. Jarrett, 1994. Antibacterial silver. Metal-Based Drugs., 1: 467-482.
[15]. Nies, D., 1999. Microbial heavy-metal resistance. Applied Microbiol. Biotechnology, 51: 730-750.