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Co-Existence of Antibiotic and Heavy Metal Resistance by Bacteria from Wound Infection

Jasmine R*[1], Daina[1], Akshyavardhini[1], Anita[1], B. N. Selvakumar[2]

1. P.G. Dept. of Biotechnology, Bishop Heber College, Tiruchirappalli-17

2. Consultant microbiologist, CSI General Mission Hospital,Trichy-3

ABSTRACT

With an intention to screen for bacterial isolates from wound infection exhibiting resistance to antibiotics, we also checked for the resistance to heavy metals, since both may be plasmid borne. Several bacteria have naturally developed tolerance to a wide range of antibiotics and toxic heavy metals. Some bacteria have also evolved mechanisms to detoxify heavy metals. Tolerance of zinc and copper by these bacteria were studied and it was found that gram positive bacteria were more resistant than gram negative bacteria. We could determine the common pattern of resistance of the isolates to both antibiotics and metals.

Key words: antibiotics; heavy metals; copper; zinc; metal tolerance

*Corresponding Author Email: jasmine_selvakumar@yahoo.com

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INTRODUCTION

Heavy metal contamination in the environment has become a serious problem due to the increase in the addition of these metals to the environment. Natural sources as well as the anthropogenic sources account for this contamination, which has become a threat to public health. Cadmium, copper and zinc are among those heavy metals that are being released to the environment[1]. In this perspective many approaches have been used to assess the risk posed by the contaminating metals in soil, water bodies etc. At present the tolerance of soil bacteria to heavy metals has been proposed as an indicator of the potential toxicity of heavy metals to other forms of biota[2]. Therefore, there is a dramatic increase in the interest on studying the interactions of heavy metals with microorganisms. Bacteria develop heavy-metal resistance mostly for their survivals, especially a significant portion of the resistant phenomena was found in the environmental strains (with or without the presence of heavy metals). One theory for bacterial heavy-metal resistance evolved is due to the use of antibiotics. For example, bacterial antibiotic-plasmids (sometime these plasmids are very big and called megaplasmid) existed in bacteria before the antibiotic era but their presence was brought into prominence by the use of antibiotics, which selected for antibiotic resistant strains. Subsequently, the range of genes carried on these plasmids (frequently associated with these heavy metal resistant determinants) was shown to extend far beyond those coding for antibiotic resistance. Similarly, heavy metals are also widespread in the environment; exert a selective pressure for the population of these plasmid-harboring bacteria. Although most of these resistances were linked to the plasmids, some are chromosomal origins. The objectives of the present study were The goals of this study were 1) to isolate the microorganisms in the orthopaedic wound infections 2) to investigate the presence and prevalence of heavy metal resistance of wound isolates taken from orthopaedic wound infected patients 3) to obtain the antibiogram pattern and 4) to determine the maximum tolerance range of the chosen metals by the isolates, thereby standardize the concentration of the metals as toxic antibacterial agent.

METHODS AND METHODS

Test Chemicals and Media

Stock solutions of copper and zinc salts were prepared by dissolving the respective sulphate salt (Sigma) in MilliQ water. Working test metal solutions were prepared by diluting the concentrated stock solutions as required, and were sterilized by filtration. All glassware was acid washed before use to avoid binding of metal. All the media used in the experiments were dissolved in MilliQ water and sterilized by autoclaving.

Specimen

Samples were collected form both the inpatients and outpatients of the orthopaedic department. Pus, aspirated fluid, a deep wound biopsy or a curetted specimen (after cleaning the wound) and tissue biopsy (from the edge of the wound) were identified by conventional methods. Antimicrobial susceptibility testing was performed by standard disc diffusion method as recommended by National committee of clinical laboratory standards[3]. All bacteria were screened for resistance to copper and zinc salts.

Determination of the Effect of Metals on Bacterial Growth

Toxicity of the selected metal to the bacterial isolates was determined using 10ug/ml concentration of the metal. 48 well sterile polystyrene microplates was used in this study as growth vessels. Sterile NB was amended with heavy metal and inoculated with exponentially growing cultures (24 h old, optical density of 0.090 at 600 nm) of bacterial isolates prepared in the same medium. Medium without metal but the bacterial inoculum (bacterial growth control) and medium with metal but without bacteria (abiotic control) served as controls. All the experiments were conducted in triplicates. Microplates were then closed with aluminium foil and sealed using additional laboratory film (Parafilm® M). The test microplates were incubated at 25ºC on an orbital shaker at 100 rpm. Bacterial growth was measured in terms of optical density at 600 nm at 0hr, 24 hrs and 48 hrs respectively.

Maximum tolerable concentrations

MTC of heavy metals

To determine the Maximum Tolerable Concentration (MTC) of the metal, several dilutions of the metal salt was prepared based on preliminary screening (1µg, 5 µ, 10 µg , 25 µg , 50 µg , 75 µg , 100 µg). To each set a bacterial culture was inoculated and plates were incubated and observed for growth by streaks on Nutrient agar plates. Strains that could not grow on 1.0 µg were termed as sensitive to the metal, while that which grew in 50 µg were further tested for higher concentration.

Antibiogram Pattern of metal treated organisms

Such metal treated bacteria were tested for antibiotic resistance following Bauer method[4]. The zones of inhibition were compared with that of metal untreated bacteria.

RESULTS & DISCUSSION

Isolation and identification of bacteria.

The collected bacteria were cultured and identified at Microbiology lab using standard protocols. The isolates obtained for this study were identified as *E.coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa sp.* and *Bacillus sp*. based on standard biochemical tests.

Growth of bacteria with metal induction curves

To find out whether metal tolerance mechanism was inducible or not, growth curves of the chosen bacterial cultures were studied. Overnight cultures were inoculated in nutrient broth containing 10ug/ml of copper and zinc sulphates. Growth curves with respect to OD600 were obtained. It was observed that there was a steady increase in growth in *S.aureus* even upto 48 hrs in the presence of Zn, but a gradual dip at later stage in the presence of Cu salt (Table 1, Figure 1), whereas in *E.coli* there was a marked decrease in growth even resembling the initial value when grown in Zn salt, while in Cu there was not much decrease (Figure 2). Unlike the other two isolates, *Bacillus sp* and *Pseudomonas sp*.demonstrated gradual increase followed by a small dip at 48hrs (Figure 3&4).

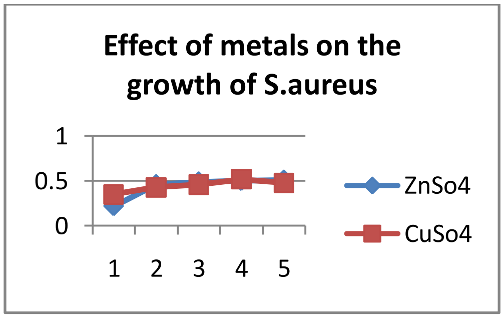

Figure 1: Growth pattern of *S aureus* in the presence of metals

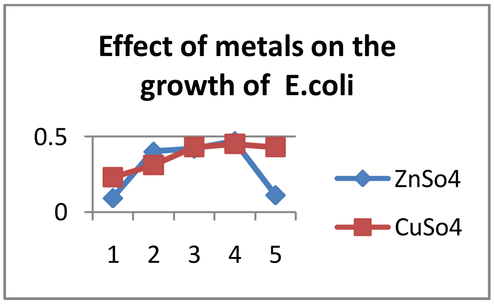

Figure 2: Growth pattern of *E.coli* in the presence of metals

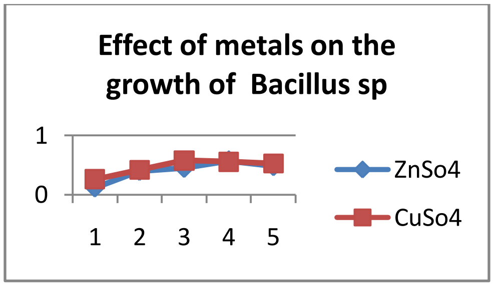

Figure 3: Growth pattern of *Bacillus sp* in the presence of metals

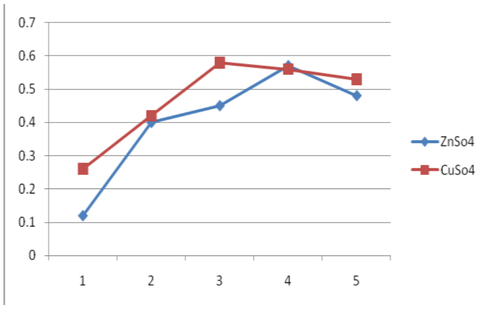

Figure 4: Growth pattern of *Pseudomonas sp* in the presence of metals

Table: 1 MTC of heavy metals

Metal con	Zinc salt				Copper salt			
	S.aureus	***E.coli***	***Bacillus sp.***	***Pseudomonas sp.***	***S.aureus***	***E.coli***	***Bacillus sp.***	***Pseudomonas sp.***
1µg	+	+	+	+	+	+	+	+
5µg	+	+	+	+	+	+	+	+
10µg	+	+	+	+	+	+	+	+
25µg	+	+	+	+	+	+	+	+
50µg	+	+	+	+	+	+	+	+
75µg	+	+	+	+	+	-	+	+
100µg	+	-	+	+	+	-	+	+

Antibiogram Pattern of metal treated organisms

Such metal treated bacteria when tested for antibiotic resistance, most of the bacteria demonstrated zones of inhibition greater than the metal untreated bacteria as shown in the table (Table 2).

Table: 2 Comparison of the zones of inhibition of metal treated and untreated bacteria

Organisms	Zones of inhibition (mm)			
	Nx-10	**IPM -10**	**AMC-30**	**Va-30**
Pseudomonas sp.	15	20	7	7
Pseudomonas sp.-metal treated	17	21	7	7
Staphylococcus aureus	7	15	10	6
S. aureus- metal treated	9	17	15	7
E.coli	8	10	6	3
E.coli -metal treated	5	14	40	-
Bacillus sp.	11	16	7	7
Bacillus sp.-metal treated	14	19	11	9

Nx-Nalidixic acid, IPM- Imipenem, AMC-Amikacin, Va-Vancomycin

Maximum tolerable concentrations

MTC of heavy metals

Among the three chosen isolates (*S.aureus, E.coli and Bacillus sp*), *S.aureus and Bacillus sp* were seen to grow upto 100 µg of both Zn and Cu salts, while in *E.coli* could tolerate Cu metal only upto 50 µg, but could tolerate 75 µg concentration of Zn salt.

Copper easily interacts with radicals, best with molecular oxygen. Its radical character makes copper very toxic (Table 2), and many organisms are more sensitive to copper[5] than *E. coli*. Copper toxicity is based on the production of hydroperoxide radicals[6] and on interaction with the cell membrane[7]. Zinc is a component in such a variety of enzymes and DNA-binding proteins, such as zinc-finger proteins, which also exist in bacteria[8] that life seems not to be possible without this redox-inactive former of tight complexes. Zinc may be complexed by various cellular components[9], and is transported by members of a variety of protein families (Fig. 1C). Biotechnology aims to create value by transforming a cheap substance into an expensive product. There are three areas for using heavy-metal resistance in biotechnology: first, adding metal resistance to a microorganism may facilitate a biotechnological process, which may or may not be linked to heavy metals. Second, heavy-metal resistant bacteria may be used for any kind of bio-mining of expensive metals, directly on ores or by recovering metals from effluents of industrial processes. Third, heavy-metal-resistant bacteria may be used for bioremeditation of metal-contaminated environments. How metal resistance can be added to a microorganism of biotechnological use depends on the amount of control one has over the process, which itself depends on the increase of value the process creates. In a highly controlled fermentor reaction, the insertion of a heavy-metal-resistance determinant into the chromosome of a particular bacterium is easily brought about by molecular genetics, if the toxic effect of a heavy metal has to be diminished. On the other hand, a sewage plant with limited control over the cleaning process probably does not allow the use of a highly modified organism. For biomining of ores, either the bacteria must be able to solubilize the respective metal directly, e.g. by reduction or oxidation, or the biotechnological transformation of another element, metal or not, is used in an indirect process. A few metals may be reduced or oxidized by bacteria, e.g. copper and iron. The indirect interaction with other elements is limited to sulfur, carbon, some metals, and the effect of the organic acids excreted by the bacteria. For recycling of metals in an industrial effluent, the value of the metal obtained must be higher than the value of the bacteria used. In most cases, the high costs of growing bacteria and the low specificity of the bacterial accumulation process make such a cleaning procedure unattractive. Drug resistance is one of the most serious global threats to the treatment of infectious diseases[10]. Metal tolerance and antibiotic tolerance behavior have revealed a very interesting pattern, where the strains that showed tolerance against the metal demonstrated tolerance against the antibiotics. This signifies an important observation regarding correlation of metal tolerance with antibiotic resistance. Statistical analysis performed revealed a positive correlation between metal and antibiotic resistance. Hence, in short there are prospects for future research on heavy metal tolerance in bacteria. Thus in an environment with multiple stresses, it is important for the organism to acquire multiple resistance for its better survival.

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