Study of mercury (II) chloride tolerant bacterial isolates from Baghmati River with estimation of plasmid size and growth variation for the high mercury (II) resistant *Enterobacter* spp.

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A total of three mercury resistant belonging to genus *Enterobacter*, *Streptococcus* and *Pseudomonas* were isolated from river banks of Baghmati in Kathmandu and were further categorized on the basis of their tolerance to mercury (II) chloride. Among all these isolates *Enterobacter* strain expressed highest degree of resistance towards Hg (II) chloride showing distinct growth in medium with upto 80 µg/ml of $HgCl_2$. Excessive slime production along with delayed pattern of growth and lower viability was observed for the isolate under increasing concentrations of Hg (II) supplemented liquid culture medium. Upon investigating total genetic content of this isolate, occurrence of plasmid with approximate 18 kb size and susceptible to mercuric chloride after plasmid curing suggests a plasmid mediated tolerance.

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Financial Support: We are thankful to Department of Biotechnology, Tribhuvan University for providing necessary funds to conduct the research.

Introduction

Frequent discharge of metallic ion containing toxic waste from industries and urban sectors contains high levels of arsenic, mercury, nickel, cadmium, lead and sulfur based byproducts [1, 9, 10, 26]. These wastes appear with vast distribution in urbanized regions in various forms and are one of the major causes of metallic pollution [22, 27]. Accumulation of these heavy metals affects the microbial ecology [3, 14] and growth pattern of microorganism in aquatic sources. Metallic toxicity and its related antibacterial effects are majorly due to oligodynamic property of metals, which presents obstacle to microbial growth. Despite these antagonistic factors, microbial adaptation system has evolved multiple mechanisms [6, 21] such as volatilization, extracellular precipitation, intake exclusion and extracellular sequestration to counteract adverse effects of these toxic compounds.

Certain environmental strains of bacteria have acquired highly sophisticated resistance mechanisms for mercury detoxification. Mainly e*nterobacter, streptococcus, staphylococcus,* and p*seudomonas* species extracted from aquatic sources [36, 37] of urban regions show high degree of tolerance against heavy metals

[5]. *Enterobacter* are frequently observed in nosocomial infection [8] and are unambiguous risk factors for infection with multidrug-resistant strains. In many bacteria, resistance to metallic salts is associated with plasmids [16]. Both chromosomally carried genes and transposable elements along with plasmids mediated xenobiotic tolerance have been found on *Enterobacter* species [5, 18]. Extreme environmental pollution and toxic industrial waste have induced variety of metal tolerance gene with reports of strain suggesting heavy metal tolerance level reaching above 100 µg/ml [26]. Excess untreated chemical wastes and sewages dumped into Kathmandu city's rivers [25] can be reflected by occurrence of such bacterial strains which is an indicator of extensive water pollution. Even though ions clearing mechanism like fractionation [17], complexing electrolyte [19, 30], and activated carbon treatment [24] have been used in treatment for removal of metal ions; adequate amount of metallic ions still seems to be released in the water sources. Generally, mercury tolerant bacteria are capable of detoxifying the mercury compounds by two sequentially acting enzymes namely, organomercuriallyase which cleaves the carbon–mercury bonds of typical organomercurials and mercuric reductase, which reducing Hg(II) into volatile and non lethal Hg(0). The counteract mechanism can be broad spectrum or specific for typical organometallic compound. Majority of such resistant strains are found to be actively expressing *mer* operon for mercury detoxification. This not only enables microbes to survive through toxic environment but also assists in removal [28, 35] of such compounds via detoxification or biosorption [2, 29, 33, 34]. Similar activities have been observed in *Enterobacter* species [15] showing considerable amount of intracellular accumulation of such heavy metals. This research article mainly focuses on Hg (II) chloride tolerance of *Enterobacter isolate* and tries to suggest a link between mercury (II) tolerance and plasmid content of the selected isolate.

Materials and methods

Isolation of mercury resistance bacteria

200 ml of water sample was collected in a sterile 250 ml glass container from banks of Baghmati River at Gausala, Kathmandu. Samples were filtered using standard Whatman filter paper. Culture tubes containing 10 ml of Nutrient Broth supplemented with (0-120) µg/ml mercury (II) chloride (Qualigens©) were used. 500 µl of filtered sample were added as inoculums in each culture tube and was incubated with shaking at 120 rpm, 37°C for 24 hours. Culture tubes showing visible growth above 10 µg/ml $HgCl_2$ were selected for further tests. Mercury (II) chloride resistant bacteria isolation and identifications were conducted by using standard biochemical tests and Genus of the bacteria was determined using interpretation of results with Bergey's manual of systematic bacteriology.

Determination of Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

The MIC for the selected isolates were determined using culture tubes containing nutrient agar medium supplemented using serial twofold dilution method and maintaining inoculum of 1 x 10^6 bacteria/ml. MBC was conducted by culturing the isolates in different concentrations of mercury (II) chloride supplemented Muller Hilton (MH) agar using spread plate culture technique. The tubes and culture plates were incubated at 37°C for 24 hours. MIC was defined as lowest concentration of mercury (II) chloride allowing no visible growth in culture tube. MBC was defined as the lowest concentration of $HgCl_2$ that results in more than 99.9% killing of the bacteria being tested.

Growth characteristics under mercury (II) chloride stress

The nutrient broth culture broth was standardized to 0.5 McFarland units by using sterile distilled water. Colony forming unit (CFU) was calculated by carpet culture on MH agar

supplemented with different concentrations of mercury (II) chloride (0-80 µg/ml). The MH agar plates were incubated for 18-72 hours at 37°C for observing growth.

Total DNA extraction and plasmid curing

High $HgCl_2$ plasmid was extracted by following Molecular cloning [23] standard extraction protocols. Alkaline lysis [23] method was implemented for plasmid DNA extraction and further purification of plasmid was done using plasmid ultrapurification kit (Bangalore GeNei© Kit). The DNA and plasmid extract was run in 1% agarose gel. Genomic DNA and plasmid content in the gel were visualized using Ethidium bromide (EtBr) (Hi-Media©) mediated DNA fluorescence and UV gel documentation unit (LLC, Gamma Scientific©). Plasmid curing [7] was performed by culturing the selected isolate in nutrient broth supplemented with EtBr of 100 µg/ml concentration followed by sub culturing into fresh nutrient agar plates. Isolates from fresh medium were further plated into nutrient agar with (25 µg/ml) and without mercury (II) chloride salt.

Statistics

Data are provided as means ±SEM, *n* represents the number of independent experiments. Differences were tested using one way-ANOVA, as appropriate. GraphPad Prism 5.0 was used for generation of figures and statistical analysis of data.

Results

Selection and identification

Selection of the bacterial isolates was purely based upon their tolerance to mercury (II) chloride in growth medium. Three distinct isolates were obtained in nutrient agar supplemented with 10 µg/ml of $HgCl_2$. Morphological analysis and biochemical tests (Table 1) along with interpretation of results using Bergey's manual of systematic bacteriology identified three isolates as *Enterobacter*, *Streptococcus*, and *Pseudomonas*.

Table 1. Bacterial isolates resistant to mercury (II) chloride. The table shows Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) for selected mercury tolerant isolates.

Bacterial Isolate	MIC	MBC
Enterobacter	45 µg/ml	80 µg/ml
Streptococcus	25 µg/ml	35 µg/ml
Pseudomonas	20 µg/ml	30 µg/ml

Determination of MICs and MBCs

Bacterial isolates were separately cultured in nutrient broth supplemented with 0-100 µg/ml of mercury (II) chloride at an increasing concentration interval of 5 µg/ml. *Enterobacter* isolates was found to show visible grow in medium up to 40 µg/ml of $HgCl_2$ whereas growth of *Streptococcus* and *Pseudomonas* were not observed above 20 and 15 µg/ml of $HgCl_2$ MBC for *Enterobacter* was obtained at 90 µg/ml whereas *Streptococcus* and *Pseudomonas* MBCs were observed at concentrations 35 and 30 µg/ml of $HgCl_2$, respectively. The MICs and MBCs results (Table 1) indicated that isolated *Enterobacter* was strongly resistant to mercury. This isolate was only selected for further studies.

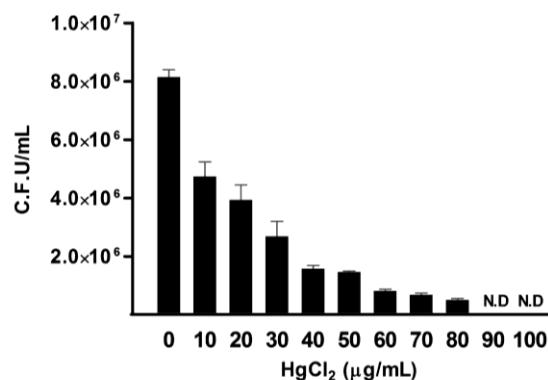

Figure 1. Effect of mercury (II) chloride on C.F.U for *Enterobacter* isolate. Isolates from various concentration of $HgCl_2$ supplemented broth were cultured by using spread plate method and C.F.U was calculated. Arithmetic mean ± SD were calculated, the figure represents average results of (n=3) independent measurement.

***Enterobacter* isolate showed $HgCl_2$ concentration dependent growth inhibition**

Growth pattern of *Enterobacter* isolate indicates that the lag phase of growth was

prolonged when cultured at nutrient agar supplemented with successively increasing concentrations of mercury (II) chloride 0-80 µg/ml in the medium. Effect of higher concentrations of $HgCl_2$ was clearly evident (Figure 1) in terms of reduction in CFU. Interestingly, presence of mercury (II) in the medium induced excessive slime production which maintained its abundance even when nutrient broth was centrifuged and washed several times with normal saline. On the contrary, growth in normal nutrient broth produced little or no slime.

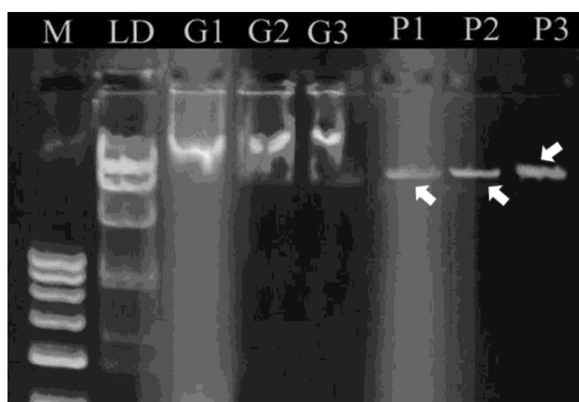

Figure 2. Extraction of total genomic DNA and plasmid from *Enterobacter* isolates. Plasmid and genomic extract was run in 1% agarose gel electrophoresis and visualized by EtBr staining. Lane 1 (L1) to Lane 8 (L8) from left to right represents Ruler DNA (M), **λ**/Mlu I digest (LD), Genomic DNA extract G1, G2, G3 along with *Enterobacter spp.* Plasmid extract P1, P2, and P3 respectively (Indicated by White arrow). Plasmid size is approximately 18 Kb with reference to LD. The white arrows indicate plasmids.

Total DNA extraction of *Enterobacter* isolate and plasmid curing

Extraction of total genetic content revealed presence of at least one plasmid common in *Enterobacter* isolates (Figure 2). Experiments were carried out in triplicates for genomic DNA and plasmid extracts. Computation of the plasmid fragment size with standard **λ** /Mlu I digest estimates it to be around 18 Kb by computing with the control of 50 Kb. Average size for the plasmid (Figure 3) was further confirmed using standard 50 kb **λ**/*Mlu* I Digest (Bangalore GeNei© Kit). Plasmid curing significantly decreased viability of *Enterobacter* isolates in $HgCl_2$ supplemented medium. Viable culture of isolates subsequent to plasmid curing was limited to 5 µg/ml of $HgCl_2$, whereas normal growth was observed in standard nutrient broth.

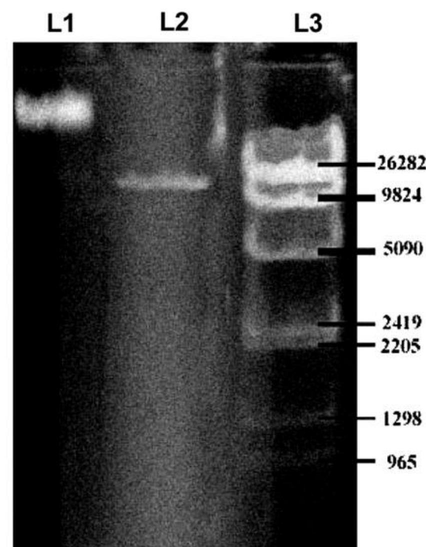

Figure 3. Plasmid extraction and estimation of size. Purified plasmid was run in 1% agarose gel electrophoresis and visualized by EtBr staining. Control DNA 50 Kb (L1), *Enterobacter* isolate Plasmid (L2), λ /Mlu I Digest (L3) [fragment size in Bp].

Discussion

Pollution of water sources as a result of various metal salts contamination [11, 12] shown to be related to development of strains capable of tolerating such metal ions [1, 4, 10, 32]. Among these contaminants mercury is one of the leading chemicals in water pollution [5] in industrialized zones. In most cases the evolutionary pattern of mutagenesis and slow genetic adaptation leads to production of resistant strains. However, unusually high level of tolerance to heavy metals ions [20] suggest a plasmid mediated tolerance strains bacteria [7]. Previous studies indicate that *Pseudonmonas* strains are presumably more competent to foreign genome [6] for their flexible genetic adaption. During the study, both *streptococcus* and *pseudomonas* isolates were found to be tolerant to mercury (II) chloride. Both of these bacteria have been known to carry plasmids and transposable elements for their mode of

tolerance [5]. However, as the focus of this research was upon high mercury (II) resistant isolate, *Enterobacter* isolate was found to grow even at concentrations upto 80 µg/ml of $HgCl_2$. High MIC (45 µg/ml) can be interrelated to the constant exposure to metal salts [26] in its native source. Mercury in its ionic form Hg^{2+} has strong antimicrobial activity mainly due to its oligodynamic effect. Mechanisms including transcytosis, intake exclusion, extracellular precipitation and volatilization are frequent in many coliforms. However, in this current investigation possibility of a plasmid mediated tolerance [7] presents the highest likelihood as suggested by the outcome of plasmid curing assay where majority of the cured cells were unable to form colony on Hg(II) supplemented medium. In addition, the plasmid extract carried out separately for the *Enterobacter isolate* yielded a plasmid of size around 18 Kb. Plasmids have been known in *Enterobacter* spp. [4, 31] for several types of tolerance. However, the exact tolerance mechanism for the metals salts is yet to be determined. Gram negative bacteria, in general, have been genetically more defiant to metal salts and *Enterobacter* is not an exception. During altered growth conditions with chemical stress of mercury (II) chloride in medium, *Enterobacter* peculiarly generated excessive slime. This might have been due to induction of excess capsule formation to counteract detrimental effects of mercury in the medium which also includes growth of microbes to from biofilm [13]. Likely, subsequent increase in Hg(II) chloride concentration depleted the CFU and also extended their regeneration time. This resulted in a diauxic growth pattern with overextended lag phase. Precise mechanism of bacteria tolerance to mercury (II) and possibility of other heavy metal resistance is a subject of further study.

Acknowledgments

We wish to express our sincere gratitude to Dr. Rajani Malla, Central Department of Biotechnology, Tribhuvan University, for providing the intellectual assistant and academic support, as well as Dr. Tribikram Bhattarai and faculty members for providing reagents and laboratory facilities.

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