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Mercury Resistance in *E. coli* Strains Isolated from Aquatic Environments of India

TANVEER A . SHAH[1], V. VERMA[1] AND ARIF ALI*

Gene Expression Laboratory, Department of Biosciences, Jamia Millia Islamia New Delhi 110025 India
[1]SMVD University, Katra, Jammu (J & K) 182121 India
[1]Baba Ghulam Shah Badshah University, Rajouri (J & K) 185131, India
E-mail : shahtanveer@yahoo.com
**Correspondence*

Abstract

Mercury resistance in bacteria (*E. coli*) at five different locations in India was studied. Physico-chemical parameters of the samples, collected from water bodies of four geographically distinct regions and hospital settings in India were checked and the samples were subsequently used for the isolation of *E. coli* strains. Out of 30 isolates of *E. coli*, eight strains showed significantly high levels of tolerance to the inorganic form of mercury i.e. mercury chloride ($HgCl_2$). Antibiotic and mercury resistance correlation was examined in the isolated *E. coli* strains. The eight highly resistant strains revealed the presence of plasmid of approximately 24 Kb.

Key words : *Escherichia coli*, Mercury resistance, Mercury chloride ($HgCl_2$).

Mercury, the sixth most toxic in a Universe of 6 million substances, exists naturally in small amounts in the environment, being the sixteenth most rare element on Earth. However, its levels have risen due to environmental contamination from human activities, such as burning coal and petroleum products. Use of mercurial fungicides in paper making, agriculture and mercury catalysts in industry, with a consequent release of mercury into air, water and on land, these activities can increase local mercury levels several thousand fold (1). As a consequence, metallic mercury is introduced into the environment, representing one of the major sources of aggression against man and the environment. Its use in seed and bulb dressings directed against bacteria and fungi on fruit trees has introduced much of the mercury that contaminates agricultural land. Therefore environmental pollution is an increacing problem both for developing and developed countries. Heavy metal resistance is now common phenotype of bacteria isolated from natural, industrial and medical environments and in most cases the resistance determinants are carried on plasmids (2). Resistance to mercury in bacteria appears to be governed by a specialized system which involves the conversion of inorganic and organic forms of mercury into metallic form (Hg^0). The Hg^{2+} resistance system has been detected in both Gram-negative and Gram-positive bacteria and is mainly located in plasmids or transposons. A few exceptions are *Staphylococcus aureus* and a marine *Bacillus* sp., which possess chromosomally located systems (3, 4). The present study was carried to evaluate the mercury resistance in bacteria isolated from different aquatic environments in India and also to examine mercury and antibiotic correlation in the isolated strains.

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Methods

The water samples used for isolation of mercury resistant *E. coli* strains were collected from various marine water sources across India.

Sampling Sites for Water Collection

The collected water samples represented five

Table 1. Physiochemical parameters of the collected water samples.

Source	Turbidity (A_{600}nm)	Temperature (C)	pH
1. Yamuna river, Delhi	Highly turbid	27	6.54 ± 0.05
2. Dal lake, Kashmir	No turbidity	14.5	6.90 ± 0.04
3. Jehlum river, Kashmir	Turbid	16	7.20 ± 0.02
4. Hindon river, Ghaziabad	Highly	24	6.54 ± 0.05
5. Safdar Jung hospital, Delhi	Turbid	20	7.32 ± 0.04

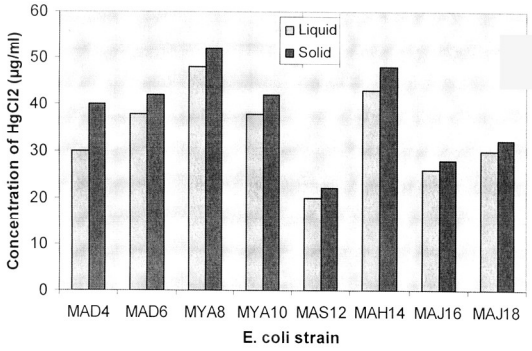

Figure 1. Maximum concentration of $HgCl_2$ tolerated by the different *E. coli* strains in liquid and solid medium.

aquatic environments from different geographical locations in India viz. Yamuna river, Delhi; Hindon river, Ghaziabad; Safdar Jung Hospital, Delhi; Jehlum river, Kashmir; Dal lake, Kashmir.

Collection of Water Samples

From five ecologically different sites, water samples were collected in sterile BOD bottles and brought to the laboratory for further processing. Water temperature, broad pH range and other physical properties were noted at the site during the sampling. The pH of the collected sample was confirmed in the laboratory using pH meter (Eutech Instruments).

Screening of Mercury Resistant E. coli Strains

All collected samples were subsequently diluted and plated on petri dishes containing growth medium i.e. Luria Agar (Hi Media, India). The initial screening of *E. coli* was done on eosin methyl blue (EMB) agar (Hi Media, India) plates. The purple colonies with greenish metallic sheen from eosin methyl blue (EMB) agar plates were selected and subjected to various biochemical tests using the biochemical test kit (Banglore Genei India) for their confirmed identification to be as *E. coli.*

Subsequently, these strains were tested for their tolerance to the inorganic form of mercury i.e. mercury chloride ($HgCl_2$) and *E. coli* strains that showed the highest tolerance level to the inorganic form of mercury were selected for further research studies.

Determination of the Tolerance Range of the E. coli Isolates to Mercury

The highest and lowest concentrations of mercury ($HgCl_2$) that could be tolerated by bacterial strains in both liquid and on solid media were determined.

Liquid Media. For determining the tolerance limits of the *E. coli* isolates to $HgCl_2$ in liquid media, the different *E. coli* isolates were grown overnight at 37 C in luria broth. Cells from overnight grown cultures were diluted 100 fold in luria broth and grown for approximately 2 hours with gentle agitation. When the absorbance at 600 nm (A_{600}) reached 0.3, these were distributed into a series of test tubes. Various concentrations of $HgCl_2$ (4—60 µg/ml) were added individually to each of the culture strains (in the various test tubes) and incubation continued at a temperature of 37 C. After 5 hours of growth A_{600} was checked and compared with that of the same strains without stress ($HgCl_2$) under the same conditions. Resistance was determined as the maximum concentrations of $HgCl_2$ that allowed normal growth of the strains.

Solid Media. Sensitivity of the strains to $HgCl_2$ on luria agar plates was tested by streaking a loopful of the culture on to solidified luria agar plates supplemented with increasing concentrations of $HgCl_2$. The highest concentration of mercury that allowed growth of different strains was recorded as resistance.

Antibiotic Resistance

The strains were tested for their resistance to the different antibiotics (ampicillin, chloramphenicol,

streptomycin, tetracycline, kanamycin and naladixic acid) qualitatively by the disk diffusion method and quantitatively by the pour plate method.

Disk Diffusion Method. The antibiotic resistance patterns of the wild type *E. cili* strains were determined qualitatively from the zones of inhibition around antibiotic disks placed equidistant on plates of luria agar medium spread with the organism.

Pour Plant Method. A quantitative estimation of the tolerance levels of the *E. coli* strains to the various antibiotics was determined by the pour plate method. Dilutions of the antibiotics were prepared in duplicate in molten and cooled luria agar medium and poured into plates. A loopful of each of the exponentially growing cultures was streaked on the plates. These were then incubated at temperature of 37 C for 24 hours. The highest concentration of the antibiotics that allowed growth was recorded as resistance to that particular antibiotic.

Plasmid Screening and Transformation

Plasmid DNA was isolated by the alkaline lysis method as described by Birnboim and Doly (5). The plasmid DNA isolated from the different strains was visualized after electrophoresis on 0.7% agarose gels in 0.5X TBE containing ethidium bromide (1 μg/ml), and the patterns were photographed with a polaroid camera. *E. coli* DH5α was used as the host for transformation of plasmid DNA isolated from the wild-type *E. coli* strains. Transformation was carried out as described by Hanahan (6). Transformants were selected on luria agar plates supplemented with different concentrations of $HgCl_2$/antibiotics to which the donor strains were resistant. Two transformants were picked randomly from each selection plate and replica plated on plates containing the same stress parameters. They were also analyzed for their plasmid content by the alkaline lysis method and compared with the plasmid profile of the wild type strains.

Results

The water samples collected from the different locations in India had various physical properties such as pH, temperature and turbidity. The Yamuna and the Hindon river samples were found to have pH 6.54 (±0.05). The samples collected from the Dal Lake, Safdarjung hospital and Jhelum river were different from each other and from above mentioned samples with pH 6.90 (±0.04), 7.20 (±0.05) and 7.32 (±0.04) respectively (Table 1).

Tolerance of E. coli Isolates to Mercurial Compounds

In the present study we isolated *E. coli* strains from water samples with special reference to mercury resistance ($HgCl_2$). A total of 30 *E. coli* isolates were collected from the five sites. The isolated *E. coli* strains when tested for their tolerance to $HgCl_2$ exhibited different resistant patterns both in liquid and solid medium (data not shown), therefore eight strains exhibiting the highest level of tolerance to the mercury ($HgCl_2$) were selected from the microbial consortia of our laboratory. The selected strains designated as MAD4 and MAD6 (Dal lake), MYA8 and MYA10 (Yamuna river), MAS12 (Safdariung hospital), MAH14 (Hindon river) and MAJ16 and MAJ18 (Jhelum river) were subjected to further study. Figure 1 illustrates the maximum levels of $HgCl_2$ that could be tolerated by the various strains in liquid and solid medium.

Antibiotic and Mercury Resistance Correlation

The widely known fact that bacterial plasmids carrying metal resistance are often known to code for antibiotic resistance. To establish this, correlation between antibiotic and mercury resistance in collected mercury resistant strains was examined, resistance pattern towards six different antibiotics i.e. ampicillin (Amp), chloramphenicol (Cm), streptomycin (Str), tetracycline (Tet), kanamycin (Kn) and nalidixic acid (Na) was established. The antibiotic resistance profile of the eight wild *E. coli* strains was determined qualitatively by the disk diffusion method and quantitatively by the pour plate method (data not shown). The antibiotic resistance phenotype did not occur with equal frequency in all the strains. The strains isolated from Dal lake and Safadarjung hospital comparatively demonstrated a greater tolerance to the tested antibiotics as compared to *E. coli* strains from the other sites. Among the eight selected strains, which showed greater resistance to $HgCl_2$, MAD4 was sensitive to

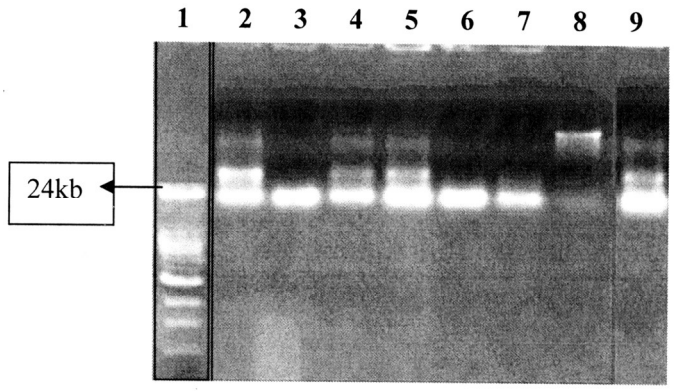

Figure 2. Plasmid DNA isolated from mercury resistant *E. coli* strains. Plasmid DNA isolated from biochemically identified mercury resistant *E. coli* (wild) strains and electrophoresed on 0.7% agarose gel. Lane 1 : λDNA/EcoRI + Hind III marker; Lane 2-9 : Plasmid DNA profile of MAD2; MAD6; MYA8; MYA10; MAH14; MAS12, MAJ16 and MAJ18.

tetracycline, MAD6 was sensitive to kanamycin, MYA10 was sensitive to naladixic acid, MAS12 sensitive to kanamycin, MAH14 was sensitive to chloromphenicol and MAJ16 isolated from Jehlum river was sensitive to streptomycin. Other strains isolated from different locations also showed resistance to tested antibiotics, but they were also sensitive to at least one antibiotic for which they were tested.

Genetics of Mercury Detoxification System

The genes for mercury resistance can be present on chromosomal DNA and on plasmids. To find out the location of genetic determinants, the following experiments were carried out.

Screening for Presence of Plasmid

Using the alkaline lysis method (5) the selected *E. coli* strains were screened for the presence of plasmid. Following plasmid DNA isolation, all the selected strains showed the presence of at least one detectable plasmid when visualized on 0.7% agrose gel. When run with a molecular marker all the eight plasmids resolved at a position, which corresponded to a size of approximately 24kb of the λDNA/*Eco*RI and Hind marker as shown in the Figure 2. Transformation of the plasmid DNA isolated from wild-type strains into the competent, plasmidless, mercury/sensitive (Hg^s) *E. coli* DH5α cells yielded transformants (data not shown) in each case on plates supplemented with different concentrations of $HgCl_2$ to which donor strains were resistant. Two transformants from each plate were analyzed for their plasmid DNA content, and visualization of the plasmid isolated from the transformants showed that they conformed to a size approximating 24kb of the λDNA/*Eco*RI + Hind III marker (data not shown), clearly identifying them to be the same as those that were transformed.

Discussion

Different water bodies including river systems, receive a variety of terrestrial wastes, sewage, and pollutants from cities and industries. Among the pollutants of serious concern, toxic metals like mercury (Hg) are important since they accumulate through the food chain leading to biological magnification, and also cause water environmental hazards.

Present study was taken to evaluate the distribution of mercury resistance in bacteria (*E. coli*) from different aquatic environments of India. Water samples for this study were collected from five different diverse ecological sites, the study was carried out on eight strains, which exhibited maximum tolerance to the inorganic mercury ($HgCl_2$) from the microbial consortia of our laboratory culture collection of 30 mercury resistant *E. coli* strains. A comparative analysis of the resistance pattern of the strains to $HgCl_2$

showed that the strains isolated from Yamuna and Hindon rivers could tolerate comparatively higher concentration of $HgCl_2$ as compared to the strains from the other sites. The highest tolerance limit was 48 μg/ml in liquid medium and 52 μg/ml on solid media (Fig. 1) exhibited by *E. coli* strain MYA8 from Yamuna river. It was also noted that the bacterial strains showed lower tolerance to mercurial compounds in liquid medium as compared to that on solid medium. A possible explanation for this difference in resistance pattern could be a better exposure of the bacteria to the mercurial compounds in liquid medium, which could result in lowering their tolerance levels as compared to that on solid medium.

Numerous studies have shown that bacteria isolated from natural water bodies carrying heavy metal resistance are usually resistant to antibiotics, both antibiotic and metal resistance can occur on the same plasmid. However, subsequently studies of these two classes of resistance markers have largely been carried out independently. While microbial ecologists have considered metal resistance as markers to environmental pollution, clinical microbial ecologists have looked only at antibiotic resistance (7).

For many years it has been assumed that the primary source of enrichment for plasmid carrying bacteria in the normal floras of humans is the consumption of antibiotics. However, clinicians are increasingly driven to consider the possibility that there may be factors in the environment apart from antibiotic consumption that results in an unusually high incidence of antibiotic resistant bacteria in healthy subjects (8). There are reports (9, 10) that mercury released from dental amalgam selects for mercury and antibiotic resistant bacteria in oral and fecal flora, in experimental settings. The selection was attributed to the genetic linkage between antibiotic resistance and mercury resistance determinants. An analysis of the antibiotic resistance profile of the 30 mercury resistant wild type *E. coli* strains showed that the strains isolated from Dal lake and Safadar Jung hospital comparatively demonstrated a greater tolerance to the tested antibiotics as compared to *E. coli* strains from the other sites. Since all the isolated strains showed notable resistance to the tested antibiotics, the ability of the plasmids to transfer antibiotic resistance along with mercury resistance was evaluated when the transformants were tested for their ability to grow in the presence of the different concentrations of antibiotics to which the donor strains were resistant. It was observed that all the transformants showed growth in the presence of one or the other antibiotic along with the mercury, clearly emphasizing the reason that there appears to be some genetic linkage between this antibiotic and mercury resistance genes. Our results also show consonance with the earlier reports of mercury and antibiotic resistance correlation reported earlier (11).

There are different mechanisms involved amongst microbes for mercury tolerance; however the most commonly found mechanism in both Gram-negative and Gram-positive bacteria involves the conversion of inorganic and organic forms of mercury into metallic form (Hg^0). This bioconversion mechanism is mediated by the genetic determenants mostly associated with plasmids. The ability to volatilize mercury from mercuric chloride or phenyl mercury acerate (PMA) seems to be a general mechanism of mercury resistance encoded by plasmid borne genes mercuric reductase (*mer*A) and organomercurial lyase (*mer*B).

In an attempt to localize the mercury resistant determinant our results confirmed the presence of this multifaceted operon, on a plasmid of approximately 24kb in size. These findings are in consonance with the earlier reports of mercury-resistant bacteria (12).

Conclusion

The isolation of mercury resistant *E. coli* isolates, which could tolerate high levels of mercury, has provided an opportunity to investigate the mechanism of mercury resistance in *E. coli*. The two most efficient strains i.e. MYA8 and MAH14 encountered in our study offer excellent potential for bioremediation and can be utilized for the amelioration of water quality and for reducing the pollution load in water bodies.

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