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Mercury Resistance in Bacterial Strains Isolated from Hospitals and Clinics

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Extensive and indiscriminate use of mercury in industries, hospitals as well as in agriculture may cause widespread environmental pollution (Misra 1992a, 1992b). Biotransformed organic mercury compounds are highly toxic to living systems (Barkay 1992 ; Summers and Silver 1978). Like other bacterial strains, disease-producing organisms are quite abundantly distributed in the environment. Hospitals and clinics may also serve as sources of these microorganisms. If these microorganisms are exposed to mercury compounds for a long time, then they may become mercury resistant (Summers and Silver 1978; Misra 1992a). In bacterial strains mercury resistance is plasmid-mediated and however, in some bacterial strains mercury resistance has been reported to be chromosomally determined (Wittle *et al* 1986). The current model of mer operon contains three or four structural genes, namely merA, merT, merP, merC or merB and two regulatory genes merR and merD. MerT, merP and merC code for Hg^{2+} transport protein. MerR codes for trans acting repressor protein. Expression of the operon is inducible and depends on the presence of Hg^{2+}, the inducer. MerA and merB gene products are mercuric reductase(EC 1.16.1.1) and organomercurial lyase (EC 4.99.1.2) respectively. Mercuric reductase reduces Hg^{2+} into Hg^{0} in the presence of NADPH and a sulfhydryl compound. Hg^{0} volatilizes out of the system due to its high vapour pressure (Misra 1992b ; Ghosh *et al* 1996).

In hospitals and clinics, infections originate from exogenous or endogenous sources. Transmission may occur directly via the hands of medical staff or indirectly via contaminated apparatus. Modern intensive care equipments like artificial ventilation are difficult to clean. Catherization procedure is of high risk of nosocomial or hospital-acquired infection. Hospital infection may be acquired from the moist environment, wards, bathrooms and kitchens. Acquisition of *Pseudomonas aeruginosa* in a hospital is rapid, and upto 30% of patients may excrete the organisms within 2 days of admission (Medical Microbiology; Greenwood *et al*, 15th edition, 1997). Although the use of mercury- based compounds are banned world-wide still the use of mercury compounds are common in India and other developing countries.

The objectives of our present study were to screen various pathogenic bacterial samples isolated from patients suffering from various ailments in different hospitals and clinics in and around Kolkata and to test those isolates for mercury resistant bacteria.

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MATERIALS AND METHODS

All chemicals and reagents used in the present study were of analytical grade (E. Merck, Germany and British Drug House, U.K.). All mercury compounds used in this study were purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A.

Ten patients were selected per day for collecting urine samples from different hospitals and clinics. 5-10 ml of urine were collected in the morning from each patients in a sterile container. Just after the collection, it was placed in an ice box for transportation and in the working laboratory, it was placed in a refrigerator at 0-4°C. Isolation of microorganisms was done immediately after getting the sample.

Table 1. Collection of urine samples in hospitals, clinics etc. from patients from Urban community with poor socio-economic background.

No. of Patients Selected/ Day	Nature of disease	Male/ Female	Age group (Years)	Sample volume (in ml)
10	Urinary tract infection	Female	45- 55	5 – 10
6	Catheter used after cerebral attack	Male	55-65	5 – 10
10	Urinary track infection after major surgery	Female	50 – 55	5 - 10

0.2 ml portion of undiluted urine samples was plated on each nutrient agar plate containing 0.1 % yeast extract and the plates were incubated for 24 hours at 35 $\pm$ 2 °C. For isolating Hg-resistant bacterial colonies, the agar plates, each containing 8.4 µg/ml of $HgCl_2$ were used and incubated for 24 hours at 35 $\pm$ 2 °C.

A number of colonies grown on nutrient agar plates was picked up and each colony was streaked on a nutrient agar plate and this operation was repeated five times to isolate pure bacterial cultures. Organisms from six such purified colonies were subsequently identified as *Pseudomonas* (5 strains) and *Staphylococcus* (1 strain) following the Bergey's Manual of Systematic Bacteriology and Difco Manual of Dehydrated culture media and reagents for Microbiological and Clinical Laboratory produces. These bacterial strains were purified to isolate pure bacterial cultures. Their mercury resistance properties were studied up to their generic level and these strains were numbered as PS_1, PS_4, UR_1 UR_2, UR_5 and ES_1.

Minimum inhibitory concentration (MIC) of $HgCl_2$ and organomercurials such as phenyl mercuric acetate (PMA), merbromine (MB), thimersol (Tm), p-hydroxy mercuric benzoate (PHMB), fluorescein mercuric acetate (FMA) were determined for all purified bacterial strains using the agar cup method. Each cup contained 0.05 ml of test solution containing a Hg-compound. Concentrations of $HgCl_2$ and organomercurials except PMA and thimersol were as follows: 3, 5, 10, 12.5, 20,

30, 40, 100, 150, 200, 250, 300, 350, 400 and 450 nmoles per cup (0.05 ml). For PMA and thimersol 1, 1.5, 2, 2.5, 3, 5, 10, 12.5, 25, 40, 100 and 150 nmoles per cup. Bacterial strains were considered sensitive if the zone of inhibition of growth by agar cup method appeared at 12.5 nmoles per cup for all materials except PMA and sensitive to PMA if at 5 nmoles of PMA per cup if zone of inhibition of growth appeared.

The following heavy metal salts were used to determine their MIC values against these organisms: lead nitrate, cadmium chloride, zinc chloride, cobalt chloride, sodium arsenate, silver nitrate, copper sulfate and potassium chromate by agar cup method against the bacterial strains. The following concentrations of these salts were used: 50, 70, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600 and 650 nmoles in each cup (0.05 ml) and the MIC values of following antibiotics against these bacterial strains were determined – Sodium Benzoyl penicillin G (Pn), Ampicillin (A), Gentamycin (Ge), Ciprofloxacin (Ci), Septran (St), Cephalexin (Ce), Streptomycin (Sm), Erythromycin (Er), Amoxycillin (Am) and Chloramphenical (Cm). Selection of antibiotic resistance or sensitivity was based on the method of Griffith 1970 (Bacterial sensitivity testing).

Typical growth patterns of the isolated Hg-resistant strain *Pseudomonas* UR_1 was determined turbidimetrically in liquid medium. Each liquid medium contained 0.31×10^{-4} M, 0.62×10^{-4} M and 1.24×10^{-4} M $HgCl_2$ and control flask without $HgCl_2$ and an overnight culture was incubated separately on a rotary shaker (200 rpm) at 37 °C. The turbidity of the bacterial broth was measured at 660 nm at every hourly interval. Growth was plotted graphically.

To determine volatilization of mercury from $HgCl_2$ containing media by these Hg-resistant and Hg-sensitive bacterial strains, five hospital isolated strains were used. In control flask 3.36 mg of $HgCl_2$ were added to 200 ml of nutrient broth containing 0.1 % yeast extract. In the experimental flasks an overnight culture of isolated strains was diluted 1:10 with nutrient broth to a final volume 200 ml and 3.36 mg of $HgCl_2$ were added. The organisms were allowed to grow for 24 hours on a rotary shaker (200 rpm) at 37 °C and control flask was also shaken similarly. Then the cells were harvested by centrifugation at 6000 x g for 10 minutes at 4 °C and washed thrice with deionised water. Weighed amount of wet cells, 1 ml of supernatant after each cell harvesting and 1 ml of control medium containing $HgCl_2$ were separately taken in 50 ml volumetric flask and digested with 5 ml of 14 N HNO_3 and 5 ml of 36 N H_2SO_4 to bring all the mercury into ionic form and the mercury contents of the solution so prepared were then measured by cold vapour atomic absorption spectrometric method using Mercury Analyzer [MA 5800D manufactured by Electronic Corporation of India (ECIL), Hyderabad] that can measure 20-200 ng of mercury present in the sample (Bradenberger *et* al 1967).

Isolation of plasmid DNA encoding mercury resistance from these bacterial strains was done using established molecular biology protocol. (Molecular Cloning A laboratory manual; Sambrook *et al* 2nd Edition 1989).

Isolated plasmids were run in 1% agarose gel and checked in transilluminator. To transform the plasmid DNA in Hg-sensitive organisms of *E.coli* XL1 Blue Strains established protocol for transformation was followed. Transformed cells were grown in nutrient agar plate containing 8.4 μg/ml. of $HgCl_2$ and plates were incubated for 24 hours at 37°C. Transformed culture grown in only nutrient agar plate without mercury was used as control.

RESULTS AND DISCUSSIONS

Table 2 represents mercury and organomercurial resistance spectra of six pathogenic bacterial strains. Among these strains, strains number PS_1 and UR_1 were highly resistant to $HgCl_2$, merbromine (MB) and thimersol (Tm). However, strains number PS_4, UR_2 and UR_5 were moderately resistant to all the mercury compounds to which bacterial strains ES_1 also was highly sensitive.

Table 2. Mercury resistance spectra of Hg-resistant hospital isolates.

Strain No.	Identified as	Mercury resistance properties (MIC)*					
		$HgCl_2$	PMA	Tm	PHMB	MB	FMA
PS_1	*Pseudomonas* sp.	350	40	50	450	200	500
PS_4	*Pseudomonas* sp.	100	5	2.5	250	100	300
UR_1	*Pseudomonas* sp.	300	25	100	400	150	300
UR_2	*Pseudomonas* sp.	150	12.5	50	100	150	150
UR_5	*Pseudomonas* sp.	100	25	50	100	150	300
ES_1	*Staphylococcus* sp.	10	1	3	30	20	30

* in nmoles per cup containing 0.05 ml.

Table 3 represents divalent heavy metal resistance spectra and antibiotic resistance spectra of five of the above mentioned microgranisms. These organisms were highly resistant to most of the tested metals. Bacterial strains UR_1, PS_4 and UR_2 were highly resistant to all other metallic compounds except cadmium chloride. Previous workers reported that mercury and organomercurial resistance in bacterial strains was associated with multiple drug resistance properties (Ghosh *et al* 1996; Sadhukhan *et al* 1997), earlier workers reported that mercury resistant bacteria were resistant to other heavy metals. Fig 1 shows the growth of mercury resistant bacterial strains, *Pseudomonas* UR_1 in liquid media containing mercury compounds. The pattern of growth of these bacterial strains in the presence of $HgCl_2$ was similar to that of control cells except that there was a distinct lag of growth. This lag phase of growth increased with the increasing concentration of $HgCl_2$ in the growth medium. Similar growth pattern was also reported by Pahan *et al* (1990); Ghosh *et al* (1996); Saouter *et al* (1994).

Table 4 shows mercury volatilization by three Hg-resistant and one Hg-sensitive bacterial strains. In the control flask without any organisms 19% of total mercury

Table 3 MIC values of some divalent heavy metal and Antibiotic resistance spectra against these bacterial strains.

Strain No.	Identified as	Heavy Metal Resistance Spectra (MIC)*								Antibiotic Resistance Spectra #
		$Pb(NO_3)_2$	$CdCl_2$	$ZnCl_2$	$CoCl_2$	$NaHAsO_4$	$AgNO_3$	$CuSO_4$	K_2CrO_4	Antibiotic
PS_1	*Pseudomonas sp.*	600	150	400	400	600	500	500	500	A,Ge,Pn,Ci,St,Ce
PS_4	*Pseudomonas sp.*	600	250	300	500	600	300	300	400	Pn,Ge,Ci,St,Ce
UR_1	*Pseudomonas sp.*	400	200	300	500	600	100	300	400	Er,Am,Pn,Ge.
UR_2	*Pseudomonas sp.*	400	200	500	300	600	100	300	500	Am,Ce,St,Pn,Ge.
UR_5	*Pseudomonas sp.*	400	150	400	200	600	500	400	300	A,Ge,St,Ce,Ci,Sm

*in nmole per cup containing 0.05 ml.

This is general characteristics of mercury resistant organisms. Antibiotic resistance was determined by standard Antibiotic paper disc method of Griffith 1970.

Table 4. Volatilization of mercury from $HgCl_2$ containing nutrient broth by Hg-resistant bacterial strains after 24 h. of. growth.

Experiment Sets	Wet weight of the cells (g)	Total Hg bound by cell (μg)	Total Hg bound per g cell mass (mg)	Total Hg retained in 200 ml broth after volatilization (mg)	Total Hg lost from 200 ml broth by volatilization (mg)	% Hg lost by volatilization
Control (without organism)	-	-	-	2.727 ±0.003	0.633 ±0.002	19
Pseudomonas sp (UR_1)	0.750 ±0.004	432 ±0.004	0.576 ±0.008	0.735 ±0.004	2.193 ±0.003	65
Pseudomonas sp (PS_1)	0.828 ±0.006	409 ±0.016	0.604 ±0.003	0.695 ±0.004	2.256 ±0.004	67
Pseudomonas sp (UR_5)	0.456 ±0.004	591 ±0.016	1.296 ±0.008	0.777 ±0.005	1.992 ±0.006	59
Staphylococcus sp (ES_1)	0.419 ±0.008	54.5 ±0.003	0.130 ±0.004	2.674 ±0.003	0.632 ±0.006	19

200 ml broth initially contained 3.36 mg Hg.
Values represent the mean ±SD for six determinations.

were lost from the medium during 24 hours of incubation at 37 °C. *Pseudomonas sp. PS1* which was highly Hg-resistant, showed high Hg-volatilizing capacity i.e. 67% after 24 hours. Weakly Hg-resistant organism such as *Staphylococcus sp* ES_1 shows similar abiochemical volatilization (i.e. 19%) like the control set under similar experimental condition. It was also clearly evident from our work that these mercury resistant bacteria had limiting capacity to volatilize mercury from mercury compound. Similar observation was also reported by Ghosh *et al* 1996; Sadhukhan *et al* 1997.

The highly mercury resistant *Pseudomonas* strains UR_2, PS_4 and UR_5 contained plasmid DNA whereas in case of highly mercury resistant *Pseudomonas* strains PS_1 and UR_1 no plasmid DNA was found. To examine whether these plasmids harboured Hg-resistance or not, they were transferred to a Hg-sensitive *E. coli* XL_1 Blue strain. The transformed Hg-sensitive *E. coli* strains became mercury resistant which were examined by streaking the culture on agar plates containing 8.4 μg/ml of $HgCl_2$.

Few colonis were obtained. These colonies were also resistant to other mercury compound at the concentrations mentioned earlier. From this experiment it can be

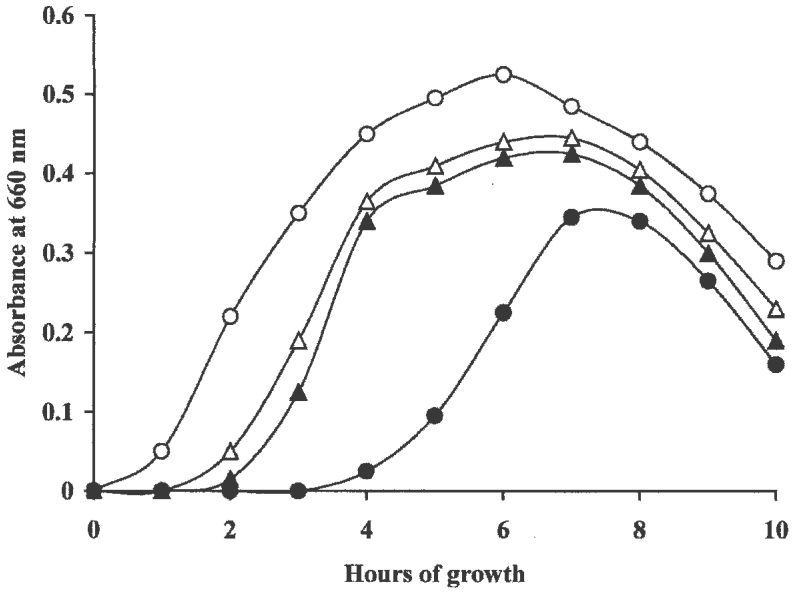

Figure 1. Growth of *Pseudomonas* UR_1 in nutrient broth with (Δ) 0.31 x 10^{-4} M, (▲) 0.62 x 10^{-4} M, (•) 1.24 x 10^{-4} M $HgCl_2$ or (O) without $HgCl_2$.

Table 5. Transformation of Hg-sensitive XL1 Blue *E.coli* strain with the plasmid DNA isolated from mercury resistant *Pseudomonas* strains.

Strains	Nature of mercury resistance	Sensitivity towards mercuric chloride ($HgCl_2$).
Pseudomonas sp ps_1	Chromosomal	Sensitive
Pseudomonas sp ps_4	Plasmid	Resistant
Pseudomonas spUR_1	Chromosomal	Sensitive
Pseudomonas spUR_2	Plasmid	Resistant
Pseudomonas spUR_5	Plasmid	Resistant

concluded that in *Pseudomonas* strains UR_2 and PS_4 mercury resistance may be plasmid determined. Although *Pseudomonas* strains PS_1 showed high mercury resistance pattern, the absence of plasmid DNA in those strains indicated that their mercury resistance pattern may be chromosomally determined.

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