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Isolation and molecular identification of mercury resistant bacteria and detection of *Escherichia coli* mercuric reductase gene from wastewater of Khowr-e-Musa, Iran

Farshid Kafilzadeh[1*], Yasamin Zahirian[1], Hossein Zolgharnein[2]

[1]*Department of Biology, Jahrom Branch, Islamic Azad University, Jahrom, Iran*

[2]*Department of Marine Biology, Faculty of Marine Science, Marine Science and Technology University, Khorramshahr, Iran*

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Abstract

Mercuric compounds are extending over large natural environment as a result of industrial pollution. Resistance to these compounds have been found in a wide range of bacterial species isolated from various environment. The aim of this study was to investigate of mercury resistant bacteria on the seashore wastewater of Khowr-e-Musa in Mahshahr area, in the south west of Iran, which one of the most important petrochemical chlor-alkali unit is located there. For this purpose, water samples were taken from wastewater of three stations. Amount of total mercury in the samples was measured using cold vapor atomic absorption spectrophotometery. Two approaches namely, conventional biochemical test and modern molecular approaches were used for identification. Mercury toxicity was measured via minimal inhibitory concentration method. *Bacillus cereus, E.coli* and *Staphylococcus aureus* were isolated and identified based on 16S rRNA gene homology, and resistance to mercuric chloride was at 400, 450 and 75 ppm, respectively. The location of *E.coli* mer operon was determined by plasmid curing. *E.coli* showed the presence of a plasmid DNA which is carries mer operon, and 1695 bp of merA gene was amplified by PCR method. The results exhibited that isolated bacteria in present study were resistant and could grow on high concentration of mercury.

* **Corresponding Author:** Farshid Kafilzadeh ✉ kafilzadeh@jia.ac.ir

Introduction

Nowadays, one of the environmental issues is the mercury pollution. Toxic trait of mercury and its accumulation in chain food result in a public health crisis in all over the world. Mercury compounds have no biological activity despite of other heavy metals such as Cu, Fe, Mn, Zn, Co, which cells are in need of them in enzyme cofactors or electron acceptor in anaerobic respiratory, nucleic acids and other cell components. What retains the stability of these metals includes specific and non-specific transfer mechanism and flow pump and carrier molecule (Nies, 1999). Physical and chemical methods such as chemical precipitation, ion exchange, reverse osmosis and superficial absorption activated carbon are the most common mercury refines processes. Usage of each of these methods has its benefits and limits (Wagner-Dobler, 2003).

Nowadays using microorganisms, heavy metals can be eliminated based on active and passive absorption (Barkay and Wagner-Dobler, 2005). Amongst microorganisms, bacteria which are the most important and diverse because of capability in stabilizing metal ion, adaptation, and being cost effective are appropriate for the process (Vieira and Volesky, 2000). Microbes especially bacteria have gain multi-mechanisms for inhibition of mercury toxicity. A gene collection called mer operon is the best known Mercury resistance system (Barkay *et al.,* 2003). The mechanisms of mercury detoxification have done via volatilization for highly toxic ionic Hg^{2+} to its volatile metallic form Hg^{0} by enzymatic reduction. Thus, the resistant bacteria can contribute to mercury removal (Griffin *et al.,* 1987). The mer operon is a genetic system consist of four or five structural genes and regulatory genes (Nakahara *et al.,* 1979), merP is a binding protein which is located on periplasmic space that link to mercury ion through two subunits. Then, merP produce a reaction with another protein in internal membrane called merT and receive mercury ion from merP (Morby *et al.,* 1995). Afterwards mercury is changed to metallic mercury via performance of mercuric reductase enzyme.

The product of merA gene is MR which is the center of mercury resistance system (Schiering *et al.,* 1991). Kafilzadeh and Mirzaee study in 2008 found that high mercury level in the environment can increase the ability of resistance to mercury among the bacterial colonies settling in the contaminated sites. Some researchers have focused on mercury resistant bacteria for bioremediation. Thus, to raise the efficiency of Hg^{2+} elimination, it is important to research more about highly resistant bacteria and bacterial mobile genetic elements, such as plasmids or transposons, which carry multiple gene encoding metal and antibiotic resistance. The present study focuses to isolate, identify mercury resistant bacteria and then to amplify *Escherichia coli* mercuric reductase gene by PCR from the very highly mercuric polluted Khowr-e-Musa.

Materials and methods

Sampling

The laboratory study was focused on the petrochemical chlor-alkali unit of Mahshahr, which located in Khuzestan province. Hence, samples were collected from three stations near sewage outfall of the Khowr-e-Musa from areas at around $30°\ 24'$ to $30°\ 25'$ N and $49°\ 07'$ to $49°\ 08'$ E. To prevent Mercury absorbed by the walls of plastic containers, samples were collected with nitric acid and potassium dichromate solution. After collection, water samples held in cooler flasks with ice bags and transported to laboratory for chemical and microbial analysis (US.EPA, 1999).

Determination of mercury levels

First, the samples were dehydrated using an oven at $103C°$ for 2h. All particles of samples were eroded to a same size. The eroded samples were digested with a mixture of 6 mL nitric acid and 2 mL perchloric acid and heated. Digested samples were filtered with 42 mm whatman filter paper (MOOPAM, 1999). Then, the amount of total mercury in the samples was measured using cold vapor atomic absorption spectrophotometery.

Bacterial counts:

Density of environmental bacteria according to bacterial species and environmental conditions is highly variable. Thus, the bacteria were counted by the total viable plate count method. First, the dilution from 10^{-1} to 10^{-10} was prepared from all samples by physiological serum. Then each prepared dilution was cultured in nutrient agar medium, containing 25 ppm Hg^{2+} nutrient agar medium and without Hg^{2+} by surface plate method. The plates were incubated at $30C°$ for 48h. After incubation, the number of colonies was counted in cultures containing Hg^{2+} and without Hg^{2+}.

Isolation, identification and mercury toxicity test of mercury resistant bacteria:

To isolate the bacteria from the water sample the primary enrichment culture method was used and directly plating on agar containing Hg^{2+} and incubated at $30C°$ for 48h. Therefore, samples were cultured on nutrient agar with the addition of 0.6 g/ml Hg^{2+} as $HgCl_2$. Bacterial colonies were capable of growth when streaked on medium plates with 500ppm mercury. Then, grown colonies were purified and identified with conventional biochemical tests and 16S rRNA sequencing.

Analysis of 16S rRNA

Genomic DNA from 18-hour culture was extracted using Bacteria Genomic Extraction Kit (cinnagen, Iran). For performing Polymerase Chain Reaction (PCR), universal primers which were capable of amplification in 16S rRNA gene have been used: 27f ($5'$ ACG GAT CCG GAT TAG CTG GTA GAG GAG $3'$) and 1492R ($5'$ GTC AAA GCT TCT AGA CTG GGA AAC TGG $3'$). PCR temperature program was done as follows: Initial denaturation $94°$ for 5 min followed by 30 cycles at denaturation $94°$ for 1 min, primer annealing $52°$ for 1 min and extension $72°$ for 2min, and final extension was $72°$ for 10min. The PCR products were purified using Gel Extraction Kit.

Single bands were observed and identified in the 1500 bp and PCR product was sent for sequencing company Takapouzist. The results of sequencing using the BLAST program software Mega4 arranged by email http://ncbi.nlm.nih.gov sequences were compared, and the bacteria were identified as the highest similarity.

Plasmid curing

This test was carried out to recognize the source of resistance against Hg^{2+} in isolated bacteria. In order to plasmid curing, species which were cultured after several times in LB broth culture without Hg^{2+}, still were capable of growing at the exposure of 20ppm mercuric chloride, were incubated in LB broth culture with non- toxic concentration of ethidium Bromide for 12h. To find out result of plasmid ccuring and growth ability, gradual dilution from bacteria were supplied in 0.85% NaCl solution and were cultured over LB agar containing 20ppm $HgCl_2$.

Isolation of plasmid and detection of merA gene

Plasmid DNA was extracted by vivantis Plasmid Extraction kit. Polymerase chain reaction merA was performed for the bacteria that cause resistance plasmid, and primers are designed for use in Gram-negative bacteria. The merA gene of size 1695 bp was amplified by the following primers: FJ $5'$ CGG GAT CCA TGA GCA CTCTCAAAATCACC $3'$ and RJ $5'$ TCC CCC GGG ATC GCA CAC CTC CTT GTC CTC $3'$ with following program: $95°$ for 5min, 30 cycle of $95°$ for 1min, $63°$ for 2min, $72°$ for 3min and $72°$ for 5min. Then, the PCR product was observed by agarose gel 0.7% electrophoresis.

Statistical analysis

Data analysis was performed using spss software and ANOVA and Duncan testes, and the significance limit was in $p<0.05$ level.

Results

The amounts of mercury contaminants in the samples were showed different levels. Accordingly, the highest level of mercury was 20.63 ± 0.46 ppm, and the lowest amount of mercury was 4.7 ± 0.42 ppm.

ANOVA, is shown significant difference between the amount of mercury in each of the three station ($p<0.05$). The logarithmic average of the number of bacteria in the medium containing mercury was (2.95 cfu g^{-1}), which is lower than the control medium that showed the logarithmic average (4.01 cfu g^{-1}).

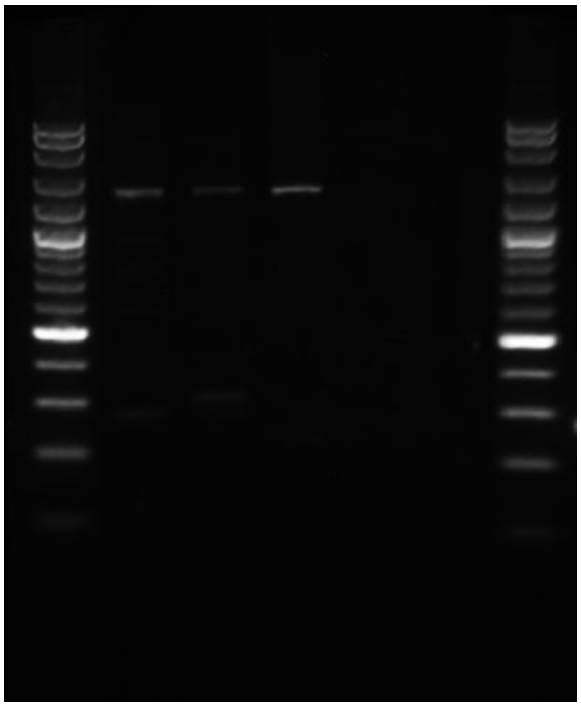

Fig. (A) Electrophoresis of PCR products for the three strains. Lane1: DNA Ladder, Lane 2&3&4: PCR amplification of 16S rRNA.

There was a significant variation ($p<0.05$) between the logarithmic values of the number of mercury resistant bacteria isolated in the different stations. Based on the results of the comparison of 16S rRNA gene sequences of three strains with the National Center for Biotechnology Information at the site, identified as: (fig. 1A) *E.coli* which could tolerate 450ppm (Hg^{2+}), *Staphylococcus aureus* and (Table 1). The results exhibited that isolated bacteria in the present study were resistant and could *Bacillus cereus* grow on high concentration of mercury. Plasmid curing, showed the presence of plasmid in *E.coli* strain which cultured after several times in culture without Hg^{2+} Still Could tolerate the exposure of 20ppm mercuric chloride. This plasmid DNA is carries mer operon. Therefore, 1695 bp of merA gene was amplified by PCR method (fig. 1B).

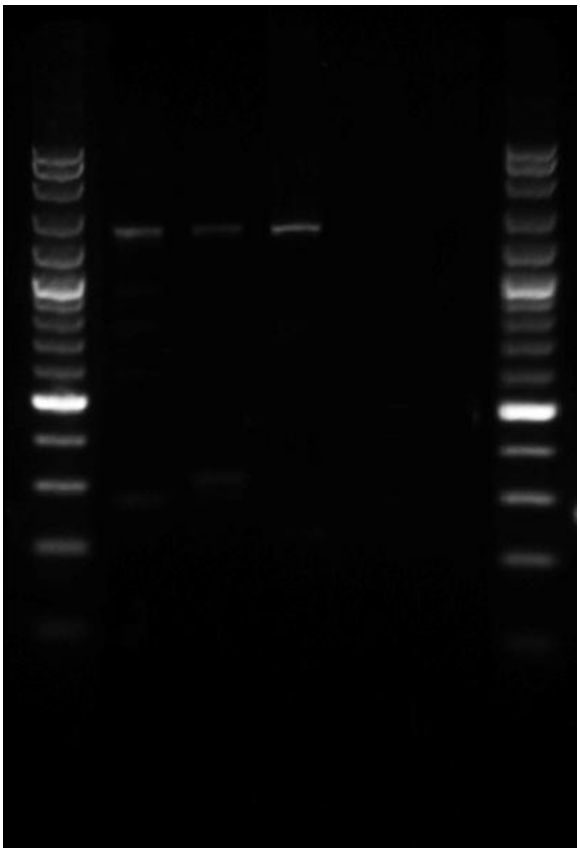

Fig. (B) Lane1: DNA Ladder, Lane 2: PCR amplification of merA gene from *E.coli* in this study, Lane3: PCR amplification of merA gene from *E.coli* R100 strain (positive control).

Discussion

The amount of mercury throughout the area in question is higher than universal standards, so that it is twenty times more than the environmental acceptable threshold (US.EPA, 1999). Conflict among results is due to condition during sampling, such as researcher's mistake or system error. According to principles of E.P.A organization, it is better to measure mercury in less than a week after sampling. In some cases error could be due to the time (Morel *et al.*, 1998). Results of the present study are compatible to results obtained in Hassan *et al.* (1998) study in

terms of distinction in MIC between broth culture and solid culture. Solid cultures have often higher MIC than broth cultures. According to Zeyaullah *et al.* (2010) it is possible that deploy of direct culture that results in real estimation of bacterial resistance against mercury or its absorption, can be true in this case.

Table 1. Minimum inhibitory concentration of mercury-resistant strains.

strain	The MIC in solid medium based on ppm	The MIC in broth medium based on ppm
E.coli	450	400
Staphylococcus aureus	75	75
Bacillus cereus	400	300

According to the results, findings of the present study which has been taken from more contaminant area can be more justified. Based on Nakano and Avila-Campos (2004), we can say that samples of current study in terms of resistance to mercury are in higher level than samples of hospital sewage and stool of Brazilian children which were in less exposure of mercury. It is relates to industrial area of Mahshahr and it is highly polluted region. Vetriani *et al.* (2005), were more successful in determination of bacteria species, therefore more precise contrast between findings of two studies can be provided. Resistance of some kind of species in such situation is due to existence of mercury in boiling water came out of chimney, and existence of sulfur reducer bacteria. Because of small amount of mercury in earth cortex, we cannot expect more resistance. The amount of mercury in the ocean, in above mentioned area, was between 7.2 to 148.4 ng/l and it is thousand times of mercury amount in the sea. It is contrastable with contaminant superficial water (Vetriani *et al.,* 2005). On the base of Zeyaullah *et al.* study in 2010, resistance of *E.coli* against mercury has plasmid origin, which is similar to findings of current research. Plasmid has been isolated from this bacterium and has been transformed to sensitive mercury cell, so existence of mer operon has been proved this way. According to Zeng *et al.* (2009), concluded that mer operon is located on the chromosome of *Pseudomonas aeruginosa* and transferable factors such as plasmid does not play role in it. Moreover, Nakano and Avila-Campos (2004) stated that mer operon from *Bacillus cereus* is located on genomic DNA and its resistance to mercury ion is concerned to chromosome. Cloning and expression of merA in transgenic bacteria and plants will be an excellent example for the bioremediation of mercury pollution (Rugh *et al.,* 1998).

Conclusion

The results depicted that *Bacillus cereus, E.coli* and *Staphylococcus aureus* were resistant and could grow on high concentration of mercury. Bacterial transferable genetic elements, such as plasmids or transposons, can transport multiple gene that encoding metal and antibiotic resistance. Metal-resistant strains may also have usage in bioremediation of metal polluted environments.

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