R-factor in *Proteus vulgaris* from ulcerative disease of fish, *Channa punctatus*

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A *Proteus vulgaris* isolated from external ulcers of the fresh water fish *Channa punctatus* showed multidrug resistance and heavy metal tolerance. The isolate from the ulcer showed resistance to chloramphenicol (Ch), nalidixic acid (Nx), streptomycin (Str) and tetracycline (Tet) with minimum inhibitory concentration (MIC) values of 750, 150, 75 and 125 μg/ml, respectively. The isolate showed growth in medium containing cadmium (Cd²⁺), up to a concentration of 2.5 mM indicating its heavy metal tolerance. Resistance to Ch, Str, Tet and Cd²⁺ of the isolate was lost after plasmid curing. Presence of plasmid DNA in the wild type and its absence in the cured *P. vulgaris* suggested that the resistance was plasmid mediated.

Ulcerative disease in fishes caused by bacteria has been reported. Due to the use of wide range of antibiotics in aquaculture, the fish pathogenic bacteria tend to develop resistance to a number of antibiotics. Information on plasmid DNA from fish pathogenic bacteria is meagre in our country. Chattopadhyay and Mandal isolated plasmid DNA from ampicillin resistant bacterial pathogens of a freshwater fish. On the other hand, heavy metal resistance was reported from clinical and environmental isolates of bacteria. In the present study a *Proteus vulgaris* has been isolated from skin ulcer of a freshwater fish *Channa punctatus* which was resistant to multiple antibiotics and heavy metal, cadmium (Cd²⁺). Genetic basis of the resistance to antibiotics and heavy metal of the isolate was determined.

Isolation and identification of the bacterium—Freshly captured freshwater fish *Channa punctatus* showed ulcers on skin at the posterior part of the body around tail. The ulcer sites were examined for pathogenic infection. For surface sterilization the fish was washed with 3% lyso1 followed by repeated washing with sterile normal saline (0.85% NaCl). Superficial tissues from the ulcer sites were removed. Tissues from the affected interior part was taken aseptically and homogenised. The homogenised sample was inoculated into sterile nutrient broth (Himedia, India) and incubated at 37°C for 18 hr. The bacterium was taken to pure culture on nutrient agar (Himedia, India) plates by dilution streak of the broth culture and for 18 hr of incubation at 37°C. The isolate was identified by standard morphological and biochemical tests. Two uninfected parts of the body were passed through similar treatments as control.

*Antibiotic susceptibility*—Antibiotic susceptibility was checked by disk diffusion method. Microbial sensitivity discs of five selective antibiotics, prepared in the laboratory, were used to find out the drug resistance pattern. Each sensitivity disc contained a single antibiotic. The amounts of ampicillin (Amp), chloramphenicol (Ch), nalidixic acid (Nx), streptomycin (Str) and tetracycline (Tet) were 25, 30, 30, 10 and 10 μg respectively. MIC values of the antibiotics were determined by agar dilution method, using nutrient agar medium and a loopfull of inoculum from an 18 hr broth culture of the bacterium, followed by incubation for 24 hr at 37°C.

*Heavy metal tolerance*—Heavy metal tolerance of the isolate was determined according to Alahmed and Yadava with some modifications. Sterile nutrient agar medium was distributed (20 ml each) in sterile culture tubes containing different concentrations of Cd²⁺ (0.5, 1, 1.5, 2, 2.5 and 2.5 mM). The tubes were inoculated with the tested microorganism (about 10⁷ cells / 100 μl in each), mixed well and poured on sterile plates. The number of colonies, grown on the plates after 24 hr of incubation at 37°C were counted.
**Plasmid curing**—Plasmid curing was carried out following Anjanappa et al., with slight modifications. The isolate was incubated at 37°C for 7 day in 2 ml of nutrient broth containing 0.1 mg/ml of ethidium bromide. The broth culture was plated onto nutrient agar media at 2, 5 and 7 day followed by an incubation for 18 hr at 37°C. After incubation, replica plates were prepared on antibiotic or cadmium containing medium to check the loss of resistance.

**Isolation of plasmid DNA and agarose gel electrophoresis**—The wild type bacteria as well as the cured one were subjected to plasmid DNA isolation following alkaline lysis method of Birnboim and Doly. Electrophoresis of the isolated plasmid DNA with tris-borate buffer system was carried out at 50 V in 0.8% horizontal agarose gel for 3 hr. The gel was stained with ethidium bromide (0.5 μg/ml) and the plasmid bands were visualized under UV-transilluminator.

A *Proteus vulgaris* was isolated from the ulcer of Channa punctatus. Tissue scrape from the healthy part of the skin did not show presence of the bacterium even after incubation for 48 hr at 37°C. Antibiotic susceptibility test revealed that the bacterial isolate was resistant (Table 1) to chloramphenicol (Ch), nalidixic acid (Nx), streptomycin (Str) and tetracycline (Tet), and sensitive to ampicillin (Amp) when standard amounts of antibiotics were used. Minimum inhibitory concentration (MIC) of the antibiotics (Ch, Nx, Str, Tet), to which the isolate was resistant, were 750, 150, 75 and 125 μg/ml respectively (Table 2).

The isolate showed resistance to cadmium (Cd²⁺) up to a concentration of 2.5 mM, while higher concentrations were inhibitory (Fig. 1).

The multidrug resistant (MDR) *P. vulgaris*, at 7 day of incubation in nutrient broth containing 0.1 mg/ml of ethidium bromide, became sensitive to Ch, Str, Tet and Cd²⁺, but remained resistant to Nx (Table 3). Agarose gel electrophoresis showed a single plasmid band in wild type while the cured one did not show any plasmid band (Table 3).

The widespread use of antibiotics in aquaculture has been followed by the emergence of antibiotic resistant bacteria causing fish disease. Plasmid mediated antibiotic resistance in fish pathogenic bacteria has been reported earlier. Aoki et al. has shown that the resistance to Ch, Tet and sulfonamethoxime (Sm) in *Vibrio anguillarum* were plasmid mediated. Later, R-plasmid conferring resistance to Ch, Str and sulfonamides were reported in *Aeromonas salmonicida*.

In the present study, the isolate contained plasmid encoded resistance to multiple antibiotics, viz. Ch, Str and Tet (Table 1) with high MIC values (Table 2).

Deposition of heavy metal salts, from industrial effluents, in the aquatic environment as well as their accumulation in biological chains could enhance the selection of heavy metal resistant bacteria. Similar to the antibiotic resistance in bacteria, heavy metal resistance was also shown to be plasmid mediated. One of the important findings of the present investigation was that the MDR *P. vulgaris* showed tolerance to Cd²⁺. The increasing concentration of Cd²⁺ resulted in the decrease in number of bacterial colonies in nutrient agar (Fig. 1). A concentration of 2.5 mM of Cd²⁺ was the highest tolerated concentration which permitted only 0.027% growth of the inoculum (Fig. 1).

In the present study, resistance to the antibiotics (Ch, Str, Tet) and the heavy metal (Cd²⁺) in *P. vul-
Table 3—Sensitivity pattern and plasmid of wild type and cured strain of P. vulgaris

<table>
<thead>
<tr>
<th>P. vulgaris</th>
<th>Sensitivity pattern</th>
<th>Plasmid</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>Resist. Ch. N x Str.</td>
<td>Present</td>
</tr>
<tr>
<td></td>
<td>Sensitive Amp. Ch.</td>
<td></td>
</tr>
<tr>
<td>Cured strain</td>
<td>Res. N x</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td>Sensitive Str.</td>
<td></td>
</tr>
</tbody>
</table>

The concentrations of Amp. Ch. N x Str and Tet were 25, 30, 10, 10 and 10 μg/ml respectively. The amount cadmium (Cd²⁺) was 0.5 mM.

Amp = ampicillin, Ch = chloramphenicol, N x = nalidixic acid, Str = streptomycin, Tet = tetracycline.

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References