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# Note

# *In vitro* susceptibility of *Pseudomonas* sp. isolated from freshwater fish to antimicrobial agents

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## ABSTRACT

Ten species of *Pseudomonas* isolated from healthy and diseased fish species such as catla (*Catla catla*), rohu (*Labeo rohita*), mrigal (*Cirrhinus mrigala*), goldfish (*Carassius auratus*), climbing perch *(Anabas testudineus*) and magur (*Clarias batrachus*) were evaluated for susceptibility to a battery of antimicrobial agents. The bacterial species used for testing were *Pseudomonas putida, P. aeruginosa, P. vesicularis, P. syringae, P. pickettii, P. compranosoris, P. alkaligenes, P. fluorescens, P. stutzeri* and *P. carboxydoflava*. The bacteria were screened for susceptibility to 31 antimicrobial agents by the Bauer Disc diffusion method. Most of the bacteria were found susceptible to oxytetracycline, gentamycin, tobramycin, amikacin, ceftriaxone, netillin, tetracycline, and amoxycillin. Majority of the *Pseudomonas* spp. tested were resistant to cephotaxime, cephalexin, cotrimoxazole, chloramphenicol, nalidixic acid, furazolidone, norfloxacin, augmentin, fluconazole, clotrimazole, cefoxilin, cephalothin, carbenicillin, ceftazidime, piperacillin, tricarcillin, amphotericin-B, cloxacillin and cefuruxime. Testing of the bacterial pathogens belonging to the genus *Pseudomonas* against gentamycin, oxytetracycline, norfloxacin, tetracycline and amikacin gave larger zones of inhibition on agar plates indicating their usefulness against Pseudomonads' disease outbreak.

Keywords: Antimicrobial agents, Freshwater fish, *In vitro* susceptibility, *Pseudomonas* sp.

Fish share a common community among aquatic vertebrates, which remain in a hostile environment loaded with various abiotic and biotic agents such as pollutants, stress factors, bacteria, virus, parasites, fungi *etc*. The incidence and outbreak of disease can be correlated to the interaction with the aquatic environment as well as with the pathogenic and non-pathogenic microflora present within the aquatic ecosystem. Biological and economic factors have been known to affect the feasibility of fish farming. Infectious diseases, in particular have been known to adversely affect the economic viability of a fish farm (Roberts and Shepherd, 1997). Representatives of several microbial genera have been implicated as pathogens of freshwater and marine fish. Continuous use of drugs as feed additives for fish may lead to development of drug resistance in the intestinal bacteria. Antimicrobial agents are used in aquaculture for controlling bacterial diseases, either as feed additives or directly into the fish pond as a prophylactic agent. The use of antibiotics in fish farms has increased extensively not only to control the various disease conditions but also to enhance production. In India, diseases due to *Pseudomonas* species has posed enormous problems to fish farmers and ornamental fish keepers. In the present investigation, *in-vitro* screening of a wide range of antimicrobial agents was carried out against, ten Gram-negative *Pseudomonas* species isolated from diseased and healthy fishes with an objective to identify some effective antibiotics.

Different species of *Pseudomonas* (*P. putida, P. aeruginosa, P. vesicularis, P. syringae, P. pickettii, P. compranosoris, P. alkaligenes, P. fluorescens, P. stutzeri* and *P. carboxydoflava*) were isolated from healthy and diseased specimens of catla (*Catla catla*), rohu *(Labeo rohita*), mrigal (*Cirrhinus mrigala*), goldfish *(Carassius auratus*), climbing perch (*Anabas testudineus*) and magur (*Clarias batrachus*) showing ulcerated skin, erosions in tail and fins. Fishes were collected from fish culture ponds of the Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar and also from local farms in Bhubaneswar, Orissa. Isolation of different species of *Pseudomonas* was done aseptically from diseased as well as healthy fish showing ulcerations, erosions and haemorrhages. The bacteria isolated were cultivated on Tryptone Soya Agar, *Pseudomonas* Isolation Agar and Brain Heart Infusion Agar (HiMedia, Mumbai). The bacteria were identified to species level by Gram staining and different biochemical tests as per Cowan *et al.* (1975) and were grown on specific media at 37 °C for 24-48 h. Results of various biochemical tests were scrutinized carefully and on the basis of the variations in biochemical properties, ten species (strains) of *Pseudomonas* could be identified and demarcated.

Different *Pseudomonas* cultures were further individually grown in Brain Heart Infusion Broth for 24 h. at 37 °C. The suspension was reinoculated on Diagnostic

Sensitivity Medium (DSM) plates by swabbing. Using sterile forceps, discs of each antibiotic (HiMedia, Mumbai) were carefully placed on the DSM agar surface at a distance of 20 mm from the edge and were sufficiently separated from each other to avoid overlapping of the zones of inhibition. The discs were tightly pressed with a sterile forceps to make complete contact with the surface of the medium and finally the plates were incubated at 37 °C for 24-48 h. The sensitivity of the bacterial pathogens were tested by the disc diffusion methods (Bauer *et al.,* 1966) with thirty-one numbers of antimicrobial agents. The antimicrobial agents tested were: cephotaxime (Ce; 30 mcg), cephalexin (Cp; 30 mcg), co-trimoxazole (Co; 25 mcg), chloramphenicol (C; 30 mcg), nalidixic acid (Na; 30 mcg), furazolidone (Fr; 50 mcg), norfloxacin (Nx; 10 mcg), oxytetracycline (O; 30 mcg), ampicillin (A; 10 mcg), augmentin (Au; 30 mcg), fluconazole (Fu; 10 mcg), clotrimazole (Cc; 10 mcg), gentamycin (G; 10 mcg), tobramycin (Tb; 10 mcg), cefoxilin (Cn; 30 mcg), cephalothin (Ch; 30 mcg), amikacin (Ak; 30 mcg), carbenicellin (Cb; 100 mcg), ceftazidime (Ca; 30 mcg), ceftriaxone (Ci; 30 mcg), netillin (Nt; 30 mcg), piperacillin (Pc; 100 mcg), tricarcillin (Ti; 75 mcg), trimethoprim (Tr; 25 mcg), sulphamethoxazole (Sx; 25 mcg), tetracycline (T; 25 mcg), amphotericin-B (Ap; 100 mcg), cloxacillin (Cx; 10 mcg), flumequine (Fm; 5 mcg), cefuruxime (Cu; 30 mcg) and amoxycillin (Am; 30 mcg). Zone of inhibition was measured after 48 h of incubation with a slide caliper. Based on the zone of inhibition, the *Pseudomonas* spp. were individually described as sensitive, intermediately sensitive and resistant to each antibiotic as per the table of recommendation proposed by the manufacturers.

The pattern of susceptibility of different *Pseudomonas* isolates to the antibiotics tested is presented in Table 1. The antibiogram revealed that the pathogens were sensitive to oxytetracycline, gentamycin, tobramycin, amikacin, netillin and tetracycline whereas resistant to cephalexin, cephotaxime, co-trimoxazole, chloramphenicol, nalidixic acid, furazolidone, norfloxacin, augmentin, fluconazole, clotrimazole, carbenicillin, piperacillin, tricarcillin, amphotericin-B, cloxacillin and cefuruxime. More precisely, *P. putida* was highly sensitive to gentamycin and amikacin, *P. aeruginosa* to netillin, *P. fluorescens* to netillin, *P. vesicularis* to gentamycin, chloramphenicol and amikacin, *P alkaligenes* to norfloxacin and ceftriaxone, *P. syringae* to gentamycin and tetracycline, *P. pickettii* to amikacin, *P. compranosoris* to gentamycin and tobramycin and *P. carboxydoflava* to cephalexin and gentamycin.

Sykes and Mathew (1976) reported that *P. aeruginosa* was sensitive to â-lactam and resistant to carbenicillin which produces cell enlargement and filament formation. In our study it was found that *P. aeruginosa* was also resistant to carbenicillin, which might be due to cell enlargement and filament formation. Johnsen (1977) observed that *P. fluorescens* was sensitive to benzyl penicillin as it utilizes benzyl penicillin as carbon, nitrogen and energy source. In our present experiment, it was noticed that *P. fluorescens* was sensitive to tobramycin, ceftriaxone and netillin. Most of the *Pseudomonas* spp. were highly sensitive to tobramycin as indicated by the zone of inhibition. Therefore, this antibiotic could be used for controlling *P. fluorescens* infection in fishes.

Kanaujia *et al.* (1998) found that *Pseudomonas* sp. were sensitive to ampicillin, chloramphenicol and oxytetracycline and resistant to streptomycin, erythromycin, nalidixic acid, bacitracin, penicillin and gentamycin, while working with mortality studies of *Macrobrachium malcolmsonii* post-larvae in hatchery conditions. We have earlier reported that Pseudomonads were mostly sensitive to tobramycin, amikacin and netillin while working with Indian major carps (Samal, 2000). Antibiotic resistance of *Pseudomonas* spp. was described by several workers (Gaman *et al.,* 1976; Sykes and Mathew, 1976; Juny and Kim, 1997; Samal, 2000; Akinbowale *et al.,* 2007). *Pseudomonas* spp. were found resistant to carbenicillin, penicillin and â-lactamase (Lowbury *et al.,* 1969; Sykes and Richmond, 1970). Acccording to Garrod and Waterworth (1969), gentamycin, tobramycin and amikacin were effective against *P. aeruginosa*. Juny and Kim (1997) reported that *Pseudomonas syringae* was highly sensitive to ampicillin, tetracycline, erythromycin and streptomycin, whereas resistant to penicillin-G and florfenicol. Akinbowale *et al.* (2007) in their study have shown that *Pseudomonas* sp. is resistant to amoxycillin, ceftiofur, cephalothin, tricarcillin, chloramphenicol, streptomycin and trimethoprim. All of their *Pseudomonas* isolates were sensitive to gentamycin and ciprofloxacin.

The results of the present study has shown that antibiotics like tobramycin, netillin, gentamycin, oxytetracycline and tetracycline could be used in aquaculture for preventing outbreak of *Pseudomonas* sp. causing diseases. Intensive aquaculture practices is presently followed in states like Punjab, Haryana and Andhra Pradesh, where the farmers/entrepreneurs generally use antibiotics as feed additives to counteract the invasion of pathogenic bacteria. In Kolleru lake area of Andhra Pradesh, generally carp culture ponds are more than 8 ha where application of sanitizers is a costlier affair and here farmers prefer applying antibiotics as feed additives when there is an onset of such type of bacterial diseases. Our present finding revealed the spectrum of antibiotics that are found resistant and sensitive to various groups of

Table 1. Antibiogram pattern of various pseudomonads isolated from fish

| Bacteria | Antibiotic group | | |
|---|---|---|---|
| | Sensitive | Intermediately sensitive | Resistant |
| *P. putida* | C, Na, O, Au, G, Tb, Ak, Ci, Nt and T | Cp, Fr, Nx, Cn, Fm and Am | Ce, Co, A, Fu, Cc, Ch, Cb, Ca, Pc, Ti , Tr, Sx, Ap, Cx and Cu |
| *P. aeruginosa* | G, Tb and Nt | C, O and T | Ce, Cp, Co, Na, Fr, Nt, A, Au, Cn, Ch, Ak, Cb, Ca, Ci, Pc, Ti, Tr, Sx, Ap, Cx, Fm, Cu and Am |
| *P. fluorescens* | Tb, Ci, Nt, Tr, Sx, T and Am | G and Fm | Ce, Cp, Ce, C, Na, Er, Nx, O, A, Au, Fu, Cc, Cn, Ch, Cb, Ca, Pc, Ti, Ap, Cx and Au |
| *P. vesicularis* | Cp, C, Nx, O, G, Au, Tb, Ak, Ci, Nt, Tr, T, Sx and Am | Co, Na and Fm | Ce, Fr, A, Fu, Cc, Cn, Ch, Cb, Ca, Pc, Ti, Ap, Cx and Cu |
| *P. alcaligenes* | Nx, G, Tb, Ak, Ci and Nt | Ce | Cp, C, Na, Fr, O, A, Au, Fu, Cc, Cn, Ch, Cb, Ca, Pc, Ti, Tr, Sx, T, Ap, Cx, Fm, Cu and Am |
| *P. stutzeri* | Co, C, O , G, Tb, Ak, Nt, T, Fm and Am | Cp, Fr, Nx, Au and Ci | Ce, Na, A, Fu, Cc, Cn, Ch, Cb, Ca, Pc, Ti, Tr, Sx, Ap, Cx, Cx and Cu |
| *P. syringae* | G, Ak and T | C, Na, O, Au, Pc and Fm | Ce, Cp, Co, Na, Nx, Ap, Fu, Cc, Tb, Cn, Ch, Cb, Ca, Ci, Nt, Ti, Tr, Sx, Ap, Cx, Cu and Am |
| *P. pickettii* | Cp,Tb, Ak, Nt, T and Fm | Au, Nx and Am | Ce, Co, C, Na, Fr, O, A, Fu, Cc, G, Cn, Ch, Cb, Ca, Ci, Pc, Ti, Tr, Sx, Ap, Cx and Cu |
| *P. compranosoris* | Cp, C, O, G, Tb, Ak, Nt and Tr | Co, Ci and T | Ce, Na, Fr, Nx, A, Au, Fu, Cc, Ch, Cn, Cb, Ca, Pc, Ti, Sx, Ap, Cx, Fm, Cu and Am |
| *P. carboxydoflava* | Cp, C, O, G, Tb, Ak, Nt and Tr | Co, Ci and T | Ce, Na, Fr, Nx, A, Au, Fu, Cc, Cn, Ch, Cb, Ca, Pc,Ti, Sx, Ap, Cx, Fm, Cu and Am |

Pseudomonads isolated from freshwater fishes. The antibiotics towards which *Pseudomonas* spp. were found to be sensitive can be used as a drug of choice for controlling such type of infections in freshwater aquaculture.

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