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# Screening of Multi-Drug and Metal Resistant Aeromonas Species from Diverse Sources

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**Abstract:** The abuse of antibiotics in the modern era, lead the microorganisms to develop resistance. Antibiotic resistance becomes the part of natural selection in bacteria which allows them to survive in different environments. Bacteria like *Aeromonas* are able to adapt to changes in the environment such as an increase in antibiotic concentration, which often results in the development of mutations allowing them to survive in unfavourable conditions. The origin of antibiotic resistance in the environment is relevant to human health and there is an urgent need for predicting emerging resistant pathogenic microorganisms. As *Aeromonas* sp. has been reported as emerging pathogen, the multi-drug resistance was screened for the *Aeromonas* isolates obtained from fish intestine, clinical and environmental sources, against commercially available antibiotics and it was found that 95% of the isolates developed resistance towards atleast one antibiotic. The emergence of antibiotic resistance in bacterial populations is a relevant field of study in molecular and evolutionary biology as well as in medical practice. The minimum inhibitory concentration of metals were performed for the isolates and it revealed that silver nitrate at 250 µM and copper sulphate at 8 mM concentration inhibited the growth of isolates. Further the metal resistance encoding genes, *sil*P and *cop*A were screened and it was found to be positive in 70% and 43% of the isolates, respectively.

**Keywords:** *Aeromonas*, Antibiotic Resistance, Metal Resistance

## Introduction

Bacteria are among the most diverse living organisms and have adapted to a great variety of environments including the human body (Pizzaro-Cerda and Cossart, 2006). *Aeromonas* spp. are water-borne and food-borne, Gram-negative rods and oxidase positive bacteria. The 9th edition of "Bergey's Manual of Determinative Bacteriology" classified *Aeromonas* into two main groups; the psychrophilic non motile and the mesophilic motile aeromonads (Parker and Shaw, 2010). *Aeromonas* have a broad host range and often been isolated from humans with diarrhoea (Ashdown and Koehler, 1993).

Sinha *et al*. (2004) in a study observed that the majority of *Aeromonas* strains exhibited a multidrug-resistance profile and this presents a significant threat to management of *Aeromonas* mediated diarrhoea. Multiple drug resistance among *Aeromonas* sp. has been reported from many parts of the world (Ko *et al*., 1996). Multiple Antibiotic Resistance (MAR) has been registered for *A. hydrophila* isolated from freshwater fish farms in association with a variety of drugs, commonly used as feed additives (Pettibone *et al*., 1996; Vivekanandhan *et al*., 2002). The universal and often indiscriminate use of antibiotics in human and animal medicine, including aquaculture, has brought serious consequences in the emergence of resistant strains of *Aeromonas* (Alvarez *et al*., 2004).

Levy (1992) stated that the over-reliance on the antibiotic agents made us to treat symptoms normally handled by our body's own immune system. The consequence to this reliance on antibiotic therapy was that bacteria developed ways to resist them. These strains reproduced and their offspring were also resistant, capable of causing infections not cured by antibiotic drugs. The increase in antimicrobial resistance poses a growing challenge in the treatment of *Aeromonas* infections (Lee *et al*., 2008).

Science Publications

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Metals are directly or indirectly involve in all aspects of growth, metabolism and differentiation (Beveridge and Doyle, 1989). Heavy metal and antibiotic resistant competence of *A. hydrophila* have been studied by many researchers (Chandra and Monica, 2011; Odeyemi *et al*., 2012). Heavy metals are stable and persistent environmental contaminants since they cannot be degraded or destroyed. Therefore, they tend to accumulate in soils and sediments (Montuelle *et al*., 1994). In Tunisia, the persistence and proliferation of antibiotics and heavy metals resistance in bacterial pathogens, belonging to the *A. hydrophila*, in aquatic environments represents a considerable public health concern (Saidi *et al*., 2013). The structural and functional characteristics of antibiotic resistance share common themes with those of metal resistance (Baker-Austin *et al*. 2006). Woods *et al*. (2009) investigated the prevalence of silver resistance genes in 172 bacterial strains isolated from both human and equine wounds. They performed Polymerase Chain Reaction (PCR) screening for 8 genes, *sil*E, *sil*RS, *sil*P, *sil*CBA and *sil*F.

## Materials and Methods

### *Sample Collection and Processing*

Fish specimens were randomly collected from fish retail outlets in sterile polyethylene bags and brought to the laboratory using an ice chest in less than an hour. The clinical diarrhoeal samples were collected from various hospitals in and around Coimbatore city, Tamil Nadu, India and transported to laboratory using Stuart's transport medium. Subsurface soil samples were collected from mangrove region of Muthupettai, Tamil Nadu, India in sterilized plastic bags and transported to the laboratory. The samples were enriched and streaked on starch ampicillin agar medium (SAA) and incubated for 24 h at 37°C. A characteristic yellow to honey coloured colonies were selected and used for further testing.

### *Isolation of Presumptive Aeromonas Isolates*

After enrichment and streaking onto SAA, honey colored colonies were selected for enzymatic tests which includes oxidase and catalase. The oxidase and catalase positive colonies were then purified by repeated streaking on the nutrient agar and were maintained in the nutrient agar slants.

### *Genotypic Identification of Aeromonads*

PCR was done for screening of the 16S rRNA and *rnp*B housekeeping genes by using genus specific primers with the expected amplicon size of 1050 bp and 410 bp respectively. The primer sequence was tabulated in Table 1. PCR of 10 µL reaction was performed with one isolate to optimize the conditions and a 15 µL reaction was performed for all the isolates. The PCR conditions followed are presented in Table 2. Each reaction was carried out using 7 µL of PCR master mix (Ampliqon, Denmark), 3 µL of nuclease free water, 1.5 µL of each forward and reverse primers (10pM) and 2µL of template DNA (50ng).

### *Antibiotic Sensitivity Test*

Antibiotic sensitivity was tested for all the isolates used in this study. The antibiotics that are commonly used for the infection control such asAmoxyclav (30mcg), Aztreonam (30mcg), Cefpodoxime (10mcg), Cephalothin (30mcg), Chloramphenicol (30mcg), Gentamicin (10mcg), Rifampicin (5mcg), Streptomycin (25mcg), Tetracycline (30mcg), Vancomycin (10mcg) were selected for the present study. Antibiotic sensitivity test was carried out by disc diffusion method (Jorgensen and Ferraro, 2009). Muller Hinton agar (HiMedia, India) was prepared, sterilized and poured onto sterile petriplates. Pure cultures grown in nutrient broth were swabbed on the MHA plates and using antibiotic disc dispenser, discs were placed on the agar surface. After the incubation at 37°C for 18-24 h, the diameter of the inhibition zone was measured and compared with the interpretative chart provided by the manufacturer.

### *Minimum Inhibitory Concentration (MIC)*

### *MIC for Silver Nitrate (AgNO3. 6H2O)*

MIC for silver nitrate was tested with Luria-Bertani (LB) agar. A wide range of silver nitrate concentrations were used to find out the MIC values. LB agar with silver nitrate concentrations like 0 µM, 10 µM, 30 µM, 50 µM, 100 µM, 150 µM, 200 µM and 250 µM were prepared by adding appropriate volume of silver nitrate from the stock. Overnight culture was diluted up to $10^{-8}$ bacterial cells/mL in sterile PBS solution. Dilutions of $10^{-6}$, $10^{-7}$ and $10^{-8}$ were inoculated as spots (5 µL) on the surface of plates using a micropipette. Plates were incubated at 37 °C for 24 h and were observed for growth.

### *MIC for Copper Sulphate (CuSO4.5H2O)*

LB agar with copper sulphate concentrations like 0 mM, 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM and 7 mM were prepared. It was allowed to cool to 45-50 °C and appropriate volume of copper sulphate from stock was added to the medium to get the required concentration. The content of the flask was mixed well and poured into sterile petriplates. The overnight culture was diluted to 10-8 bacterial cells/mL in sterile PBS solution. Dilutions of $10^{-6}$, $10^{-7}$ and $10^{-8}$ were inoculated as spots (5 µL) on the plates with copper sulphate using micropipette. Plates were incubated at 37°C for 24 h and were observed for bacterial growth.

Table 1. Primers used in this study

| S. No. | Genes | Primer sequence | Base pairs |
|---|---|---|---|
| 1 | 16S rRNA | F-5′ CAGAAGAAGCACCGGCTAAC 3′<br>R-5′ TTACCTTATTACGACTTCAC 3′ | 1050 |
| 2 | *rnp*B | F-5′ TGGGCAATCGCTGCTTCGT 3′<br>R-5′ AGGTCGGAGTCGGCCTGTAA 3′ | 400 |
| 3 | *sil*P | F 5′- AGTGCAACACAACAAC 3′<br>R 5′- ACTTTCTCTGCACGGA 3′ | 1200 |
| 4 | *cop*A | F 5′- CTTTACGGACTTTTACCCGCC 3′<br>R 5′- GCGGCGGCCGCCTTTGGGAAGTTGAAAAC 3′ | 1300 |

Table 2. PCR conditions for the amplification of genes

| | | PCR conditions | | | | | |
|---|---|---|---|---|---|---|---|
| S. No. | Genes amplified | Initial denaturation (°C) | Denaturation (°C) | Annealing (°C) | Extension (°C) | Final extension (°C) | No. of cycles |
| 1 | 16sr RNA | 95°/5min | 94°/30sec | 52 °/30sec | 72°/1min | 72°/5min | 30 |
| 2 | *rnp*B | 95°/5min | 94°/30sec | 54.5°/30sec | 72°/1min | 75°/5min | 30 |
| 3 | *sil*P | 96°/4min | 96°/20sec | 54.2°/20sec | 72°/2min | 72°/5min | 35 |
| 4 | *cop*A | 95°/5min | 95°/1min | 50.7°/40sec | 72°/1min | 72°/10min | 35 |

### *Detection of Metal Resistance Gene*

PCR amplification of *sil*P and *cop*A was performed by using specific primers. The primers used in this study and reaction conditions were tabulated in Table 1 and Table 2.

## Results

### *Incidence of Aeromonas from Various Sources*

The incidence of *Aeromonas* spp. was recorded in the intestine of marine fish, soil and clinical (diarrhoeal) sources. A total of 130 samples were processed and 88 samples i.e., 68% showed positive for *Aeromonas*. Of the positive *Aeromonas* isolates, 79% were from fish intestine, 53% from soil and 31% isolates were of clinical origin (Table 3 and Fig. 1).

### *Antibiotic Sensitivity Assay*

Multidrug resistance was shown by most of the isolates. Maximum level of resistance was shown to cephalothin by most of the isolates and least percentage of resistance was shown towards aztreonam. All the isolates were sensitive to chloramphenicol and gentamicin. *Aeromonas* isolates obtained from fish, of about 68%, 61%, 76% and 75% were found to be resistant to amoxyclav, cefpodoxime, cephalothin and vancomycin, respectively. Soil isolates of about 94%, 78%, 83% and 83% were found to be resistant to amoxyclav, cefpodoxime, cephalothin and vancomycin, respectively. About 100%, 82%, 91% and 100% of clinical isolates were found to be resistant to amoxyclav, cefpodoxime, cephalothin and vancomycin, respectively). With regard to amoxyclav, cefpodoxime, cephalothin and vancomycin, higher frequency of antibiotic resistance was recorded among the clinical isolates.

### *Resistance Pattern*

The isolates exhibited 29 different resistance patterns. Among the total isolates, 95% showed resistance to atleast one antibiotic. Two of the isolates obtained from fish showed resistance towards a single antibiotic. The F55 isolate showed resistance towards a maximum of seven antibiotics. Four of the isolates (F56, S70, C83 and C84) were found to be resistant towards six antibiotics with 3 different resistance patterns. About 17% of the isolates developed resistance against five antibiotics with 5 patterns of resistance. Further 33% of the isolates showed resistance to about 4 antibiotics with six patterns and about 26% of the isolates shown resistance to three antibiotics with 9 different patterns for the antibiotics used. The multiple antibiotic resistance is prominent among fish isolates when compared with the soil and clinical isolates.

### *MAR Index of the Isolates*

The MAR index value of all the isolates ranged from 0.2 to 0.7. MAR index of 0.2 to 0.3 was exhibited by 40% of the isolates. MAR index of 0.4 to 0.5 and 0.6 to 0.7 was exhibited by 54% and 6% of the isolates, respectively. The MAR index in the range of 0.2 to 0.3 was shown by 41%, 28% and 37% of the fish, soil and clinical isolates, respectively. About 46%, 67% and 45% of the respective fish, soil and clinical isolates showed the MAR index ranging from 0.4 to 0.5. Very few isolates showed the MAR index ranging from 0.6 to 0.7, which includes 3%, 6% and 18% of the fish, soil and clinical isolates, respectively. About 10% of the sensitive isolates were recorded among fish isolates. None of the soil and clinical isolates exhibited MAR index <0.2.

Table 3. The percentage of incidence of *Aeromonas* spp.

| Samples | No. of samples | No. of samples positive | Percentage of incidence |
|---|---|---|---|
| Fish | 75 | 59 | 79 |
| Soil | 34 | 18 | 53 |
| Clinical | 35 | 11 | 31 |
| Total | 130 | 88 | 68 |

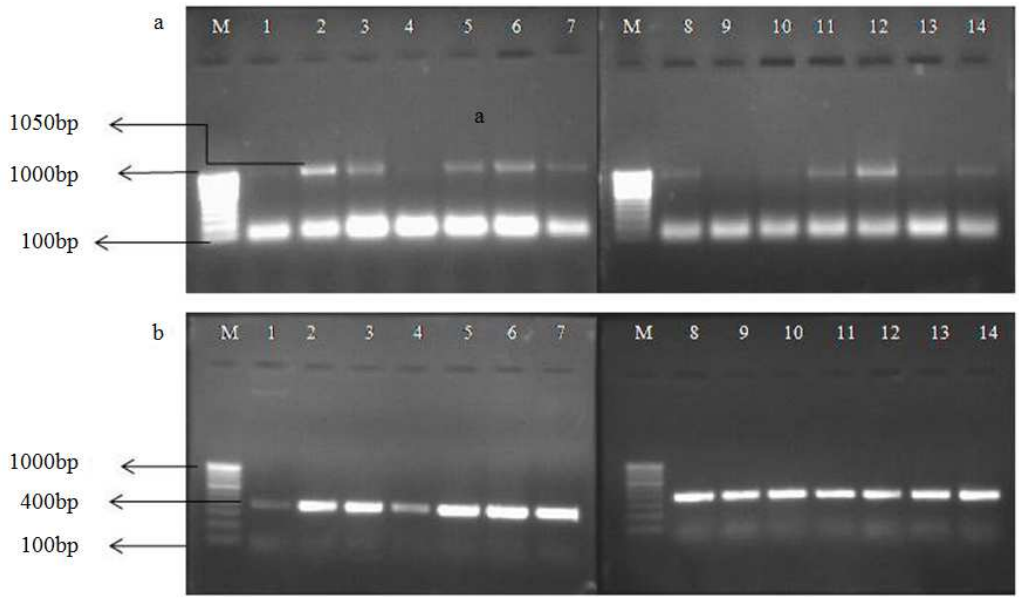


Fig. 1. Lane M- Marker 100-1000 bp. 1a-amplification of 16S rRNA gene, amplicon-1050bp; 1b-amplification of rnpB gene, amplicon-400bp; Note: Distinct bands indicate the amplification of 16S rRNA and *rnp*B genes of *Aeromonas* isolates

## *Minimum Inhibitory Concentration of Metals*

### *MIC of Silver Nitrate*

The inhibition of bacterial growth by silver nitrate was observed for different concentrations such as 0 µM, 10 µM, 30 µM, 50 µM, 100 µM, 150 µM, 200 µM and 250 µM. The LB agar without silver nitrate was used as a control. At 250 µM concentration, none of the isolates exhibited the resistance to silver nitrate. About 14% showed resistance to 200 µM concentration of silver nitrate, which comprises 11%, 18% and 15% of isolates isolated from fish, soil and clinical sources, respectively. At 150 µM concentration of silver nitrate, 49% of isolates showed resistance among which, 52%, 50% and 33% of fish, soil and clinical isolates, respectively. Whereas, at 100 µM and 50 µM concentrations of silver nitrate, 92% and 98% of resistant isolates were observed. However, the minimal concentrations of silver nitrate (10 µM and 30 µM) do not interfere with the growth of the isolates.

### *MIC of Copper Sulphate*

Inhibition of bacterial growth was observed on LB agar medium supplemented with 0 mM to 7 mM of copper sulphate concentrations. None of the test isolates showed resistance to 7 mM concentration of copper sulphate. At the concentration of 6 mM, 25% of the isolates showed resistance to copper sulphate, among which 26%, 23% and 21% from fish, soil and clinical isolates, respectively. About 63% of the isolates (63% of fish and soil isolates and 70% of clinical isolates) showed resistance to 5 mM concentration of copper sulphate, whereas at 4 mM concentration of copper sulphate, 97% of the isolates exhibited growth. As the lower concentrations of copper sulphate (1 mM, 2 mM and 3 mM) do not have much effect, hence all the isolates exhibited the growth on LB agar plate.

### *silP Gene*

The gene coding for silver resistance (*sil*P) was screened in all the isolates which is of 1200 bp. About 62 isolates (70%) were found to be conserved with this gene. Of which, about 81%, 61% and 27% of isolates from fish, soil and clinical isolates, respectively were conserved with *sil*P gene. The *sil*P gene was highly conserved among the fish isolates when compared to soil and clinical isolates. The presence of *sil*P gene do not have complete role in existence of silver resistance among the isolates screened for varying concentrations of silver nitrate except for some isolates. The presence of resistance gene and the development of silver resistance were not in correlation in all the isolates. Few of the

isolates posses the *sil*P gene but it could not resist the higher concentrations of silver and fewer shows the resistance even at higher concentrations of silver without the presence of *sil*P gene, which indicates that some other components in silver resistance gene cluster may plays vital role in development of resistance towards silver.

### *copA Gene*

The presence of copper resistance (*cop*A) was screened in all the isolates used in this study. Of the 88 isolates screened for *cop*A gene coding for copper resistance (1300 bp), 38 isolates were found to be conserved with this gene. The *cop*A gene was highly conserved among the fish isolates when compared with soil and clinical isolates since, among 43% existence, 51%, 17% and 45% was conserved in fish, soil and clinical isolates, respectively. As like the case of silver resistance, the presence of *cop*A gene do not have complete role in existence of copper resistance among the isolates screened for varying concentrations of copper sulphate except for some isolates. So the complete cluster of copper resistance genes should be studied for determining the exact component which favours the microbes to resist copper.

## Discussion

### *Incidence of Aeromonas Isolated from Various Sources*

In the present investigation the prevalence of *Aeromonas* spp. was recorded in marine fish intestine, soil and clinical (diarrhoeal) sources. A total of 130 samples were processed and about 68% showed positive for *Aeromonas*. Higher prevalence of *Aeromonas* spp. was observed in fish samples when compared to other samples used in this current research, which indicates the opportunistic nature of *Aeromonas* spp. and it was also a normal flora of fish intestine. It was reported that fish may also be a vehicle for pathogenic bacteria naturally occurring in aquatic environments referred to as indigenous or derived from polluted waters and or from post capture contamination, storage and handling. Joseph *et al*. (2013) screened for the occurrence of *Aeromonas* spp. in tropical seafood, aquafarms and mangroves of Cochin coast in South India and they recovered 11% of *Aeromonas* spp. by 16S rDNA sequence analysis. In the present study about 53% of *Aeromonas* spp. was recorded in soil samples, which indicates aeromonads are ubiquitous in occurrence. The variability in the prevalence among West coast and east coast regions of South India may be due to many reasons like river flow and anthropogenic activities.

In the current research 31% of the diarrhoeal samples were found to be contaminated with aeromonads. The prevalence of *Aeromonas* spp. among clinical isolates were found to less when compared with other sources. This was supported by Oberhelman and Taylor (2000), who reported that the isolation rate of *Aeromonas* in many developing countries may range from 5 to 28% in clinical isolates. Similarly, the occurrence of *A. hydrophila* in acute gasteroenteritis among children was reported in the Coimbatore region, Tamil Nadu by Subashkumar *et al*. (2004), where the clinical isolates were collected in the present study. Of the 216 samples they collected, (10%) were positive for *A. hydrophila*.

### *Antibiotic Sensitivity*

In recent years development of resistant or multidrug resistant pathogens has become a major problem in India and many countries (WHO, 2013). Bacterial resistance is closely associated with the use of antimicrobial agents in clinical practice. The aeromonads have been regarded as universally resistant to penicillins (penicillin, ampicillin, carbenecillin and ticarcillin) for quite a long time. In the present study the utmost resistance was found to be developed against cephalothin by the isolates and least resistance was shown towards aztreonam. All the isolates were found to be sensitive to chloramphenicol and gentamicin. The F55 isolate obtained from fish showed resistance towards a maximum of seven antibiotics. Multidrug resistance was shown by most of the isolates. With regard to amoxyclav, cefpodoxime, cephalothin and vancomycin, higher frequency of antibiotic resistance was recorded among the clinical isolates. All the isolates were found to be sensitive to chloramphenicol and gentamicin.

### *Minimum Inhibitory Concentration of Metals*

Although some heavy metals are important and essential trace elements, at high concentrations most of them can be toxic to microbes. Silver *et al*. (1989) reported that most of the bacteria developed resistance mechanisms in order to survive the high concentrations of metals in the environment. Miranda and Castillo (1998) isolated antibiotic and metal resistant *Aeromonas* isolates from polluted and unpolluted waters.

In the present study, the minimum inhibitory concentration of silver nitrate towards the *Aeromonas* isolates used in this study is 250 µM. Among the isolates screened the clinical isolates have shown constant resistance till 150 µM, which indicates the prevalence of high silver resistance isolates from clinical source. The minimum inhibitory concentration of copper sulphate towards the *Aeromonas* isolates used in this study is 7 mM. From the results it is evident that high copper resistant isolates were predominant among the clinical and fish isolates. Several of the silver-resistant strains have been collected from silver-treated patients at burn centres, where these strains have sometimes caused outbreaks (Pirnay *et al*., 2003). Pike *et al*. (2002) screened for silver resistance on MHA containing 50

μM, 200 μM, 300 μM and 500 μM AgNO3 and their results correlate with the results of present study.

In the present investigation further silver (*sil*P) and copper (*cop*A) resistance genes which are of 1200 bp and 1300 bp, respectively were screened in all the isolates. The *sil*P gene was conserved in 70% isolates and *cop*A gene in 43% of the isolates. In both the cases of *sil*P and *cop*A resistance genes, higher prevalence was noticed in fish isolates when compared with the other isolates.

## Conclusion

The genus Aeromonas is one of several medically important genus that have become an increasingly troublesome group due to its pathogenesis in human and in aquatic life. The emergence of antibiotic resistance in bacterial populations due to abuse of antibiotics is a major problem in the current situation. In the present study metal resistance of Aeromonas isolates were recorded from diverse sources. This study will be helpful to distinguish the virulent strains from normal Aeromonas flora.

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## Conflict of Interest

The authors declare that they have no conflict of interests.

## Author's Contribution

**Ramasamy Amsaveni:** Participated in all experiments, coordinated the data-analysis and contributed to the writing of the manuscript.

**Muthusamy Sureshkumar:** Participated in all experiments, coordinated the data-analysis and contributed to the writing of the manuscript.

**Joseph Reshma Mary:** Participated in performing molecular experiments.

**Umapathy Indra:** Participated in writing this research paper.

**Govindasami Vivekanandhan:** Designed the research plan and organized the study.

## Ethical Statement

The authors declare that the manuscript has not been submitted to more than one journal for simultaneous consideration, has not been published previously, none of the data have been fabricated or manipulated.

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