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Microbial Screening of Antibiotic Residues in Soil and Water of Cultured Fish Farms in Tamil Nadu

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Abstract

Background: Aquaculture is one of the main protein sources in the world. Due to the intensification in aquaculture, the occurrence and the spread of disease is more. To control and treat the disease in aquaculture, farmers started using antibiotics in improper manner. This leads to the deposition of antibiotic residues in water and soil, which later transformed to fish that makes them resistant to that particular antibiotic. It was later transformed to other animals and humans, which makes them resistant to that antibiotic. Henceforth there is a need to screen the presence of antimicrobial residues in soil and water.

Methods: In this study, microbiological methods were used for screening antimicrobial residues. For microbial screening, *Bacillus subtilis* and *Escherichia coli* were used as a marker microorganism. Totally 75 samples of soil and 75 samples of water were screened for antimicrobial residues.

Result: The results revealed that 8%, 4% and 13.33% of pond soil samples and 10.66%, 6.66% and 21.33% of pond water samples were contaminated with oxytetracycline hydrochloride, florfenicol, sulphadimethoxine and ormetoprim residues. This review clarifies that microbial screening has a unique advantage of economical and a simple method that can be used as a preliminary method for screening antibiotic.

Key words: Antimicrobial residues, Fish, Marker microorganism, Microbial screening, Soil, Water.

Introduction

Aquaculture is a rapidly growing and important source of animal protein in human diets, particularly in low- and middle-income countries (LMICs). Globally, surge for animal source nutrition is being met with a transition to increasingly intensive animal production systems (Steinfeld *et al.,* 2006; Van Boeckel *et al.,* 2015). In this context, non-therapeutic antimicrobial use may serve to increase growth and substitute for good animal husbandry practices (Schwarz *et al.,* 2001; Van Boeckel *et al.,* 2017).

Inadvent use of antimicrobials in food producing animals and human beings, enhances antimicrobial resistance (Laxminarayan *et al.,* 2013). Emerging incidence of antimicrobial resistant pathogens in aquatic farm animals also increase treatment failure rates, undermining sustainable food animal production and animal welfare. Aquaculture conditions utilizing antimicrobials may serve as reservoirs for antimicrobial resistance genes, providing routes for human and animal exposure to antimicrobial resistant bacteria (Heuer *et al.,* 2009; Cabello *et al.,* 2013; Aedo *et al.,* 2014; Larsson *et al.,* 2018; Lulijwa *et al.,* 2020).

Prolonged use of antibiotics over their normal dosage leads to some harmful effects on aquatic organisms which will cause adverse effects in both human and animal health too. These adverse effects include growth reduction and indirect effects of antibiotic resistance (OECD, 2019). Most of antiinfection agents are not totally metabolized in people and animals, a high level of drugs is released into water and soil through city wastewater, sewage slime and biosolids that are habitually used to flood and prepare agrarian terrains (Bouki *et al.,* 2013; Daghrir and Drogui, 2013; Wu *et al.,* 2014).

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Microbiological method is the basic method used for the analysis of the sample for the presence or absence of the antimicrobial residues which depends on the principle of inhibition of growth of sensitive bacteria by residues when there is an inhibitor in the test samples (Aerts *et al.,* 1995).

This study will portray on the predominance of antibiotic residues in both soil and water from fish culture ponds in three districts of Tamil Nadu and also portray whether the antibiotic residues in soil and water are within the MRLs or not. This will offer to propose rules concerning approaches and guidelines of antimicrobial deposits control.

MATERIALS AND METHODS

The study was conducted during the year 2019-2022 at the Pharmacovigilance Laboratory for Animal Feed and Food Safety, TANUVAS, Chennai, Tamil Nadu.

Sample preparation and experimental design

A total number of 150 pond soil and pond water samples were collected from commercial freshwater fish farms from Chennai, Thanjavur and Thiruvarur districts of Tamil Nadu. From these 150 samples, 75 samples were pond soil and 75 samples were pond water. These samples were analysed for oxytetracycline hydrochloride, florfenicol, sulphadimethoxine and ormetoprim residues. Totally, 450 samples were analysed for antibiotic residues.

Extraction of pond soil/pond water samples for antibiotics

The chemicals and solvents which were used for the extraction of antibiotic residues from pond soil and pond water samples were of analytical grade. Extraction was done as per the method described by Kodimalar *et al.,* (2018), Pietron *et al.,* (2014), Pietron *et al.,* (2013) for oxytetracycline hydrochloride, florfenicol, sulphadimethoxine and ormetoprim residues with some minor changes.

Culture medium preparation

Nutrient broth and nutrient agar were used as culture media obtained from HIMEDIA. As per the manufacturer's instructions, nutrient broth of about 1.3 g was weighed and dissolved with 100 ml of distilled water in a conical flask. It was then disinfected in an autoclave at 15 mm Hg of pressure and 121°C of temperature, for 15 min, which was cooled to about 50°C. To this autoclaved nutrient broth medium, 0.002 g of bromocresol purple indicator was added.

Bacterial strain preparation

The freeze-dried bacterial strain of *Bacillus subtilis* and *Escherichia coli* were procured from Microbiological Type Culture Collection (MTCC) and were activated based on the instructions given by MTCC. Single colonies obtained from petri dishes (Fig 1 and 2) were separately inoculated into 5 ml nutrient broth using a sterile loop and incubated in hot air oven at 37°C for 18-24 hrs. Then the broth suspension was adjusted to a concentration approximately equal to 0.5 McFarland standard equivalents to 1.5×10^8 CFU/ml (Kilinc and Cakli, 2008).

Microbial screening analysis of pond soil/pond water samples

Microbiological tube test method with an indicator microorganism were followed for screening the presence or absence of antimicrobial residues in pond soil and pond water samples. The indicator microorganism and the antibiotic residues screened were shown in the Table 1.

Microbiological tube test method

Procedure

1.8 ml of nutrient broth with pH marker bromocresol purple was drawn in a test tube. To that test tube 0.1 ml of sample and 0.1 ml of test microorganism was pipetted and merged properly. Both positive control (culture and nutrient broth) and negative control (nutrient broth) were also kept. All the test tubes were incubated in a hot air oven at 37°C for 18-24 hrs. The test tubes that turned yellow after the incubation were recorded as negative and those that remained purple were recorded as positive for antimicrobial residues (Jambalang, 2012).

RESULTS AND DISCUSSION

Microbial analysis of pond soil and pond water samples

The results of oxytetracycline hydrochloride, florfenicol, sulphadimethoxine and ormetoprim residues in pond soil and pond water samples by microbial screening using *Bacillus subtilis* and *Escherichia coli* as the test organisms

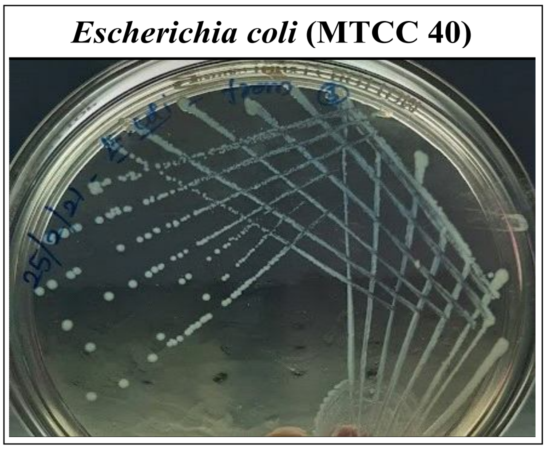

Fig 1: *Escherichia coli* (MTCC 40).

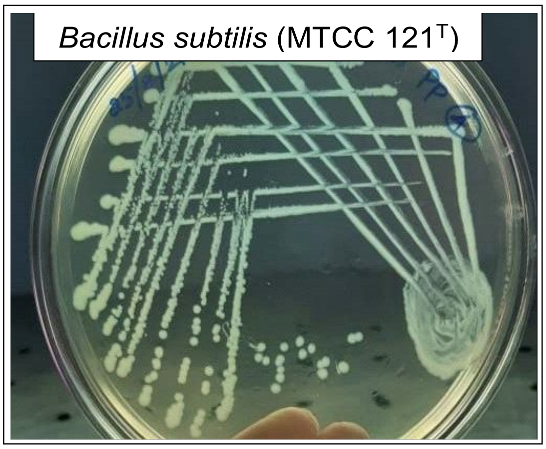

Fig 2: *Bacillus subtilis* (MTCC 121^{T}).

Table 1: Indicator microorganisms used for screening antibiotic residues.

Antibiotic residues	Indicator microorganism
Oxytetracycline hydrochloride	*Bacillus subtilis*
Florfenicol	*Escherichia coli*
Sulphadimethoxine and ormetoprim	

are presented in the Fig 3, 4 and 5 and Tables 2, 3 and 4. A total number of 150 samples in which 75 samples were pond soil and 75 samples were pond water, were analysed for residues and among the same, 6 pond soil samples (8%) and 8 pond water samples (10.66%) were found to be positive for oxytetracycline hydrochloride and for the antibiotic florfenicol 3 pond soil samples (4%) and 5 pond water samples (6.66%) were found to be positive and for the antibiotic sulphadimethoxine and ormetoprim 10 samples (13.33%) were found to be positive in pond soil and 16 samples (21.33%) were positive in pond water. Overall, 14 samples (9.33%) were found positive for oxytetracycline hydrochloride residues, 8 samples (5.33%) were found positive for florfenicol residues and 26 samples (17.33%) were positive for sulphadimethoxine and ormetoprim residues out of 150 samples.

Cengiz *et al*. (2010) reported that 50% samples of soil were positive for antibacterial substances which were related to tetracycline group. It was higher than the results of the current study. Tetracycline was able to contaminate and remain in soil for long time because of their adsorptive character. Ibraheem (2012) described that 62% of tetracycline, 25% of doxycycline, 12.5% of chlortetracycline residues were present in the water samples. This result was contrary to the present findings as only a smaller percentage of samples were found contaminated with oxytetracycline hydrochloride.

The present results were contrary to the findings of Zhao *et al*. (2016) who reported that 33.3% of soil samples were positive to amphenicol groups. The higher percentage of positive samples were due to frequent use of the antibiotic. Similarly, He et al. (2014) also reported that the percentage of positive samples for sulphonamide were high at 48%. Wei (2012) reported that more than 90% of the animal waste water and water resource samples were found to contain ciprofloxacin, enrofloxacin and florfenicol residues that were

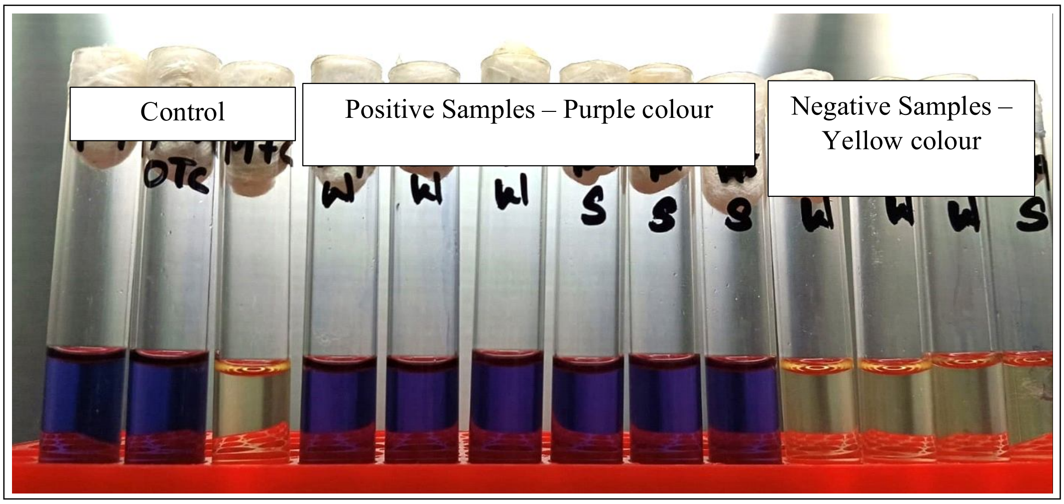

Fig 3: Screening of Oxytetracycline hydrochloride residues in pond soil/pond water samples by microbiological method using *Bacillus subtilis* as indicator microorganism.

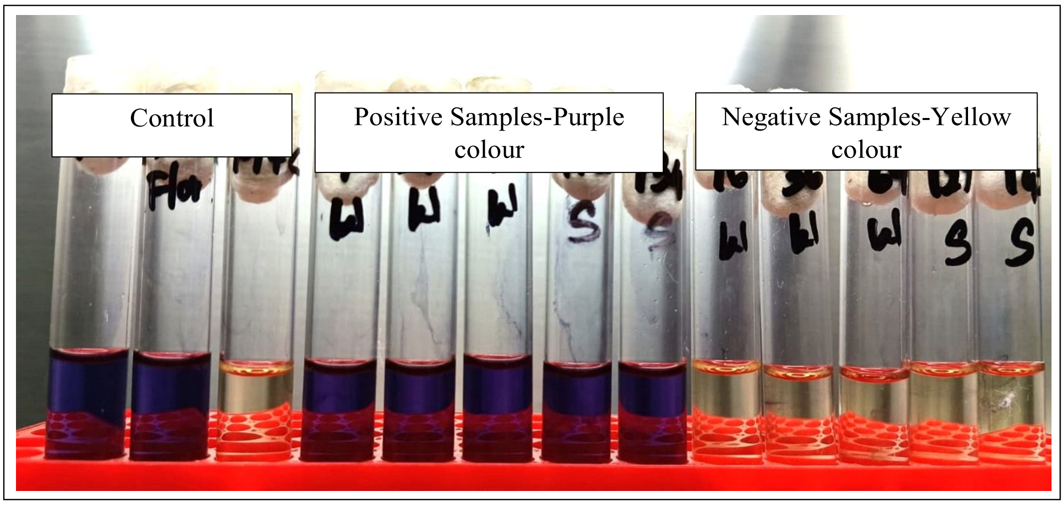

Fig 4: Screening of Florfenicol residues in pond soil/pond water samples by microbiological method using *Escherichia coli* as indicator microorganism.

also completely contradictory to the results obtained in the present study. This higher percentage of occurrence of antibiotics in water was due to the ability of the antibiotic to enter into soil, sediments, *etc*.

The LOD was found to be 50 µg/kg for oxytetracycline hydrochloride and 100 µg/kg for florfenicol, sulphadimethoxine and ormetoprim which is below the accepted maximum residue level (MRL) for the antibiotics set by European Union. The data were subjected to statistical analysis by Chi- square test and there existed a non-significant difference between pond soil and pond water for all the three antibiotics and the results are shown in Tables 2, 3 and 4 .

The lower level of positive samples of antibiotics in soil in the present study indicates that the usage of antibiotics for performance promotion in the areas of study has been much lower than the earlier reports which might be due to increasing awareness of the farmers on the after effects of continuous usage of antibiotics. However, as antibiotics like tetracycline can remain in soil for longer duration, it is more prudent to work towards zero level contamination of the pond soil.

Kabir *et al*. (2004) reported that the variation in the occurrence of antibiotic residues in present study in comparison with former studies could be attributed to a

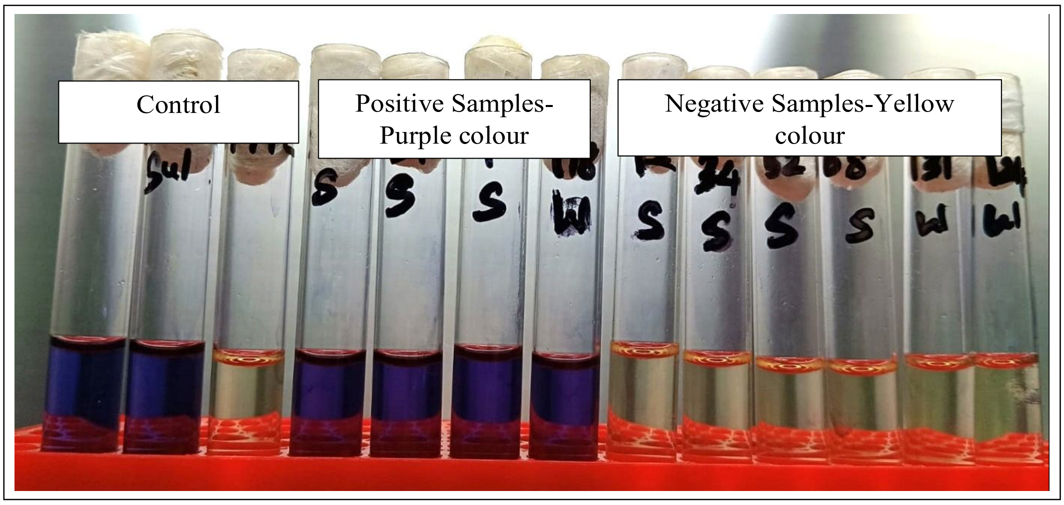

Fig 5: Screening of sulphadimethoxine and ormetoprim residues in pond soil/pond water samples by microbiological method using *Escherichia coli* as indicator microorganism.

Table 2: Microbial screening of pond soil/ pond water using *Bacillus subtilis* (MTCC 121^{T}) for oxytetracycline hydrochloride.

Sample	Antibiotic	Number of samples	No. of positive samples	% of positive samples	Chi-square value
Pond soil	Oxytetracycline	75	6	8	0.32^{NS}
Pond water	hydrochloride	75	8	10.66	

NS- Statistically non- significant at 5% level (P>0.05).

Table 3: Microbial screening of pond soil/ pond water using *Escherichia coli* (MTCC 40) for florfenicol.

Sample	Antibiotic	Number of samples	No. of positive samples	% of positive samples	Chi-square value
Pond soil	Florfenicol	75	3	4	0.63 ^{NS}
Pond water		75	5	6.66	

NS- Statistically non- significant at 5% level (P>0.05).

Table 4: Microbial screening of pond soil/ pond water using *Escherichia coli* (MTCC 40) for sulphadimethoxine and ormetoprim.

Sample	Antibiotic	Number of samples	No. of positive samples	% of positive samples	Chi-square value
Pond soil	Sulphadimethoxine	75	10	13.33	1.67^{NS}
Pond water	and ormetoprim	75	16	21.33	

NS- Statistically non- significant at 5% level (P>0.05).

difference in fish species, sampling, location, time of collection and type and level of antibiotics used, variations in experimental conditions and the sensitivity of the method used for analysis.

However, the results of the present study indicate that the usage of antibiotics in the areas under study were much lower than the earlier reports quoted from other parts of the world which is appreciable and the same needs to be taken forward through appropriate measures to ensure zero usage.

CONCLUSION

The microbiological assay procedure developed could be utilized for residue screening because of its sensitivity and affordability with cheap cost which allows screening of soil and water for residues; it should be adopted in our country with the aim of reducing the risk of antimicrobial residues in fish.

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Conflict of interest: None.

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