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Effect of Electromagnetic Fields on *Aeromonas hydrophila* Isolated from Cultured *Nile tilapia* (*Oreochromas niloticus*)

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A total of 31 *Aeromonas hydrophila* isolates from cultured Nile tilapia (*Oreochromas niloticus*) were investigated for the effect of different frequencies of electromagnetic fields (EMFs). The isolates were investigated for their susceptibility to fourteen antimicrobial agent and (2.9 X 10²CFU/ml) *Aeromonas hydrophila* viable count was introduced into the water of seven aquaria (each contains 25 fish), the aquaria then exposed to different strengths of electromagnetic fields and the bacterial content was evaluated in the water samples after 5,15.30 and 60 minutes. The total psychrotrophic count was also determined in the fish flesh after their storage in the ice for 0,24,72,120 and 168 hours. Most of the isolates were multidrug-resistant. The number of viable *Aeromonas hydrophila* was significantly decreased upon exposure to alternative low-frequency electromagnetic field (20 hz) when compared with the control and the other EMFs exposed aquaria. Psychrotrophic count of tilapia was significantly low in the electromagnetic treated Nile tilapia fish. The implementation of electromagnetic field as a new control impairing the growth of pathogenic *Aeromonas hydrophila* among Nile tilapia may be one of the potential measures and alternative approach to antimicrobials. However, further investigations should be implemented.

Key words: *Aeromonas hydrophila*, Nile tilapia, electromagnetic fields.

Nile tilapia (*Oreochromas niloticus*), is an outstanding fish among the global aquaculture and the most profitable fish among the tilapia species. *Aeromonas hydrophila* is a major cause of bacterial infections affecting warm water fish especially tilapia (Oreochromis niloticus). It can cause motile Aeromonas septicemia (MAS) in fish, both in commercial production systems and in natural waters. MAS is stress-related, and conditions such as poor water quality, overcrowding and rough handling make fish more susceptible to the bacteria. Infected fish frequently exhibit small pinpoint hemorrhages at the base of fins or on the skin, distended abdomens, and protruding eyes. Internal signs include fluid in the abdomen, swollen liver and spleen, and distended and fluid filled intestines. The disease can produce significant losses in the aquaculture industry because of reduced growth and unmarketable appearance of infected fish **(Pachanawan *et al*., 2008).**

Antibiotics have been mixed with feed for oral administration for treatment and prevention of bacterial infections in aquaculture and drugs in the same classes have been used for medical treatment in humans. Particular concern is that

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improper and over-use of antibiotics can lead to development and distribution of antimicrobial resistance among aquatic bacterial pathogens, including *Aeromonas hydrophila* that could transfer to humans (Lukkana *et al*., 2011).

The public health hazards related to antimicrobial use in aquaculture include the development and spread of antimicrobial resistant bacteria and resistance genes, and the occurrence of antimicrobial residues in products of aquaculture. The greatest potential risk to public health associated with antimicrobial use in aquaculture is thought to be the development of a reservoir of transferable resistance genes in bacteria in aquatic environments from which such genes can be disseminated by horizontal gene transfer to other bacteria and ultimately reach human pathogens. Moreover, the antimicrobials lead to drug residues in the treated fish, besides having a negative impact on the environment (Aly, 2013). Therefore, our research team since many years pay a big concern about the elaboration of new methods for controlling bacterial infections with special reference to immune-stimulants and probiotics as well as vaccination (Aly *et al*, 2008; 2010; 2013) , especially in view of constraints of standard antibiotic therapy confronted with the expansion of resistant.

The effects of electromagnetic fields (EMFs) on the biological functions of living organisms represent an emerging area of interest. Bacteria have also been used to study the effects of electromagnetic fields on the functional parameters (cell growth and viability) and antibiotic sensitivity depending on physical parameters of the electromagnetic field (frequency and magnetic flux density) applied, the time of the exposure, and/or the type of bacteria cells used (Segatore *et al*., 2012).According to these considerations, the possibility of application of the appropriately patterned magnetic fields deserves special attention in light of the risk that antimicrobial resistance poses to public health.

The aim of this study was to first, isolate and identify *Aeromonas hydrophila* from tilapia fish (*Oreochromas niloticus*), water and fish feed as well as to determine the antimicrobial profile of the isolates. Second, evaluation to the influence of different frequencies of Electromagnetic fields (EMFs) on *Aeromonas hydrophila* isolates in the water of fish aquaria and cultured tilapia fish through the determination of the total psychrotrophic count in the fish-flesh of control and electromagnetic treated Nile tilapia.

MATERIALS AND METHODS

Bacteriological examination

Isolation and identification of pathogenic bacteria:

31 *Aeromonas hydrophila* isolates were recovered from Nile tilapia exhibit the typical signs of Motile Aeromonas Septicemia (MAS); small pinpoint hemorrhages at the base of fins and on the skin, distended abdomens, fluid in the abdomen, swollen liver and spleen, and distended and fluid filled intestines. Dilutions of 1 ml from intestinal content of cultured fish and the artificial food filtrate) and liver swabs were inoculated on Tryptic Soya Broth then on Tryptic Soya Agar (Gibco); Brain Heart Infusion Agar (BioTeC); and MacConky agar (BioTeC). Separate colonies were cultured into the R-S agar media (Shotts and Rimler 1973). Individual colonies were randomly taken and preserved in Tryptic Soya Broth +15% glycerol +2% agar. The isolates then biochemically confirmed using API® 20 E (Biomerieux®, Crapponne, France). All the isolates were genetically confirmed to be the hydrophila species by using conventional polymerase chain reaction technique for the detection of species-specific primers 16S rDNA1 and 16S rDNA2 (Chu and Lu, 2005)

Antimicrobial susceptibility testing:

The resistance of 31 *Aeromonas hydrophila* isolates to different antimicrobials was determined by disk diffusion on The Mueller–Hinton agar (Difco). Fourteen antimicrobial agents were selected to represent different classes of antimicrobials relevant for therapy in human and animal medicine. Based on the distributions of the inhibitory zone diameters and, where available, recommendations from the Clinical and laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards) (CLSI/NCCLS, 2005a), break point values were used to separate the sensitive isolates from the resistant. The concentration of antimicrobials in the disks and the inhibition zone break point values of the resistance were given; where isolated *Aeromonas hydrophila* were tested for the resistance to

kanamycin (KAN), oxytetracycline (OTC), nalidixic acid (NA), ciprofloxacin (CIP) , sulphamethoxazole/ trimethoprim (SXT), chloramphenicol (CHP), amoxicillin(AMO), carbenicillin (CAR), enrofloxacin(ENR), erythromycin (ERY), gentamicin (GEN), kanamycin(KAN), streptomycin (STR), tetracycline (TET) . All the disks were purchased from Oxoid. The disk diffusion assays were done according to the recommendations of (CLSI, 2005 a & b).

Electromagnetic Field Exposure

Seven glass fresh-water aquaria (24 X 60 X 40 cm) filled with freshwater and equipped with thermo-regulating and filtration systems (water temperature 23°C, Ammonia level was 0.1, PH was 8.5). First aquaria kept without fish (groups 1) and 150 Nile tilapia, (average body weight of 150 g), were equally and randomly distributed to the other six glass fresh-water aquarium (groups 2-7, each of 25 fish). Then, 2.9 X 10^2 CFU/ml *Aeromonas hydrophila* viable counts were introduced into the seven aquaria. The first two aquaria were used as control and didn't expose to any electromagnetic fields, EMFS (first aquarium without fish). The other six aquaria (groups 3-7) were then exposed to different strengths of electromagnetic fields generated by Hemholtz coil system and measured by HIn3550 magnetic field monitor. The third aquarium (group 3) was exposed to direct low frequency EMFS (24-40 hz) while the rest of aquaria (group 4-7) were respectively exposed to different alternative EMFS (20, 100, 1000, 10000 hz). The total viable number of inoculated *Aeromonas hydrophila* was counted in the water of each aquarium at 5, 15, 30 and 60 minutes of EMFS exposure.

Determination of the bacterial count

The bacterial content was evaluated in the water of treated and control aquaria at minute 5, 15, 30, and 60 minutes.1 ml was analyzed for its concentration by measuring the optical density (OD) spectrophotometrically (Hitachi U-2810 Spectrophotometer) at wavelengths (»max)where the maximum absorption occurred.

Keeping quality tests

Determination of shelf-life

Based on the findings of Electromagnetic Field Exposure experiment, the experimented Nile tilapia of group 2 (control) and group 4 (20 hz electromagnetic treated groups) 25 fish per group, 150 g average body weight), were collected by end of the experiment and transferred immediately in sterile plastic bags on ice to the laboratory. Fish of both groups were stored in an ice container after mixing with crushed ice that was replaced daily during the storage period (7 days). The total psychrotrophic count was determined in the fish flesh of the collected tilapia of the two experimented groups after their storage in the ice for 0, 24, 72, 120 and 168 h (25 fish sample/each storage period). Ten grams of the fish-flesh were transferred into a sterile blender with 90 ml of sterile 10% peptone water. The blender was operated at a high speed (14000 rpm) for 2 minutes. The mixture was kept for 6 minutes at room temperature and decimal dilutions(10^{-1}– 10^{-6}) were prepared. One ml from each dilution of the previously prepared suspension was inoculated into duplicate plates, and then 10 ml of standard plate count agar was poured into each plate. The inoculated plates were carefully shaken, left to solidify and then incubated at 20°C for 48 h. The total psychrotrophic count was calculated according to (Thatcher and Clark 1975).

RESULTS

Bacterial strains

A total of 31 *Aeromonas hydrophila* isolates were revealed among the collected samples; 20(64.5%) isolates were recovered from the intestinal samples and 11(35.5%) from liver samples respectively.

Antimicrobials susceptibility

The results of antibiotic susceptibility of the tested strains for various antimicrobials were shown in (Table 1). The strains showed high resistance level to most antibiotics used in our study. *Aeromonas hydrophila* isolates from intestinal samples were resistant to oxytetracycline, streptomycin, carbenicillin and amoxicillin were 40 %,40 %, 38 %, 37 % respectively, while *Aeromonas hydrophila* isolates from liver samples were resistant to oxytetracycline, kanamycine, cholorompheniсol and streptomycin were 80 %, 70 %, 70 %, 60% respectively. Most of the isolates were multidrug-resistant (resistant to at least 3 or more different classes of antimicrobials). Resistance to ciprofloxacin was not detected.

The effect of different frequencies Electromagnetic Fields

The results of the effect of high and low frequencies Electromagnetic Fields were shown in (Table 2) and Fig.1. Bacterial count after 5 minutes of exposure were the lowest in the water of aquarium/group 4(0.5×10^2), which exposed to alternative low frequency electromagnetic field (20hz), while the highest count (3.3×10^2) were in aquarium/group 3, which exposed to direct low frequency electromagnetic field (24-40 hz) and aquarium/group 6, which exposed to alternative high frequency electromagnetic field (1000 hz). Bacterial count in the water after 15 minutes of exposure were the lowest(0.1×10^2) in aquarium/group 4, which exposed to alternative low frequency electromagnetic field (20 hz), while the highest (3.5×10^2) were in aquarium/group 7, which exposed to alternative high frequency electromagnetic field (10000 hz). Bacterial count in the water after 30 minutes of exposure were the lowest(1.5×10^2)in aquarium/group 4, which exposed to alternative low frequency electromagnetic field (20 hz), while the highest (4.8×10^2)were in aquarium/group 7, which exposed to alternative high frequency electromagnetic field (10000 hz). Bacterial count in the water after 1 hour of exposure were the lowest(2.2×10^2) in aquarium 4, which exposed to alternative low frequency electromagnetic field (20 hz), while the highest (5.6×10^2) were in aquarium 7, which exposed to alternative high frequency electromagnetic field (10000 hz).

Keeping quality test

Determination of shelf-life

Shelf-life was determined by the total psychrotrophic count in the fish-flesh of Nile tilapia in control and the selected electromagnetic treated groups (group 3) during ice-storage for 7 days (Table 3). The mean total psychrotrophic count was in general higher in the control group than the electromagnetic treated groups. The electromagnetic treated groups at the beginning, showed mean total psychrotrophic count of 00.11

Table 1. Antimicrobials susceptibility of *Aeromonas hydrophila* species

Bacterial isolates	Antibiotics and antimicrobial resistance (%)													
Source	TET	STR	KAN	GEN	ERY	ENR	CAR	AMO	CHP	SXT	CIP	NA	OTC	KAN
Intestinal isolates (20 isolates)	14	40	9	5	13	13	38	37	20	20	0	30	40	20
Liver isolates (11 isolates)	18	60	11	5	21	27	43	40	70	40	0	10	80	70

kanamycin (K AN), oxytetracycline (OTC), Nalidixic acid (NA), ciprofloxacin (CIP) , Sulphamethoxazole/ Trimethoprim (SXT), chloramphenicol (CHP), amoxicillin(AMO), carbenicillin (CAR), enrofloxacin(ENR), erythromycin (ERY), gentamicin (GEN), kanamycin (KAN), streptomycin (STR), tetracycline (TET)

Table 2. Comparison of *Aeromonas* count in the water of EMFs treated and control aquaria

bacterial count before starting	control 0	Control ° 0	24-40hz* 0	20hz** 0	100hz** 0	1000hz** 0	10000hz** 0
added bacteria	2.9×10^2	2.9×10^2	2.9×10^2	2.9×10^2	2.9×10^2	2.9×10^2	2.9×10^2
bacterial count after 5 minutes of exposure	2.8×10^2	3.2×10^2	3.3×10^2	0.1×10^2	3.1×10^2	3.3×10^2	2.7×10^2
bacterial count after 15 minutes of exposure	3.3×10^2	3.5×10^2	3.3×10^2	0.5×10^2	3.3×10^2	3.4×10^2	3.5×10^2
bacterial count after 30 minutes of exposure	3.9×10^2	4.0×10^2	3.4×10^2	1.5×10^2	3.6×10^2	4.1×10^2	4.8×10^2
bacterial count after 1 hour of exposure	4.1×10^2	4.3×10^2	3.7×10^2	2.2×10^2	3.8×10^2	4.3×10^2	5.6×10^2

* :direct electromagnetic wave; ** :alternative electromagnetic waves; °control: control with fish

X $10^3 \pm 0.13$ and after 24 hrs was 00.68 X $10^3 \pm 0.53$. By time, the mean total psychrotrophic count significantly increased after 72 hrs to become 6.10 X $10^3 \pm 0.43$, and although the count in this group become greatly increased at 120 and 168 hrs to be 13.56 X $10^3 \pm 0.36$ and 97.93 X $10^3 \pm 0.39$ respectively but still better than the control (non EMF exposed) group.

DISCUSSION

This study aimed to find an alternative control to replace antimicrobials for the treatment of *Aeromonas hydrophila* infection in Nile tilapia aquaculture. Antimicrobials selected for the present study were used commonly in the treatment of *Aeromonas hydrophila* infection; Based on the antimicrobials profile, *Aeromonas hydrophila* strains conferred from the present study were resistant to most of the tested antimicrobials, as an increase in antibiotic resistance of the genus *Aeromonas* has been reported (Albert *et al*., 2000).The observations regarding the activity of tetracycline, chloramphenicol, sulfonamides, quinolone and aminoglycosides are comparable to the results obtained by several other investigators, the present study results agreed with (Ko *et al*., 1996) who found that most of *Aeromonas* strains are resistant to the commonly used antibiotics such as tetracycline, trimethoprim and chloramphenicol. (Goni-Urriza *et al*., 2000) reported tetracycline-resistant *Aeromonas* species in 49% of the isolates. Also, (Jones and Wilcox, 1995) reported resistance of *Aeromonas* to sulphamethoxazole/trimethoprim. While (Sartor *et al*., 2013), detected fluoroquinolone resistant *Aeromonas* strains following leech therapy. However, our results disagreed with (Aravena-Román *et al*., 2012), who found that most *Aeromonas* strains are susceptible to trimethoprim-sulfamethoxazole (TMP-SMX), fluoroquinolones, second and third generation cephalosporins,

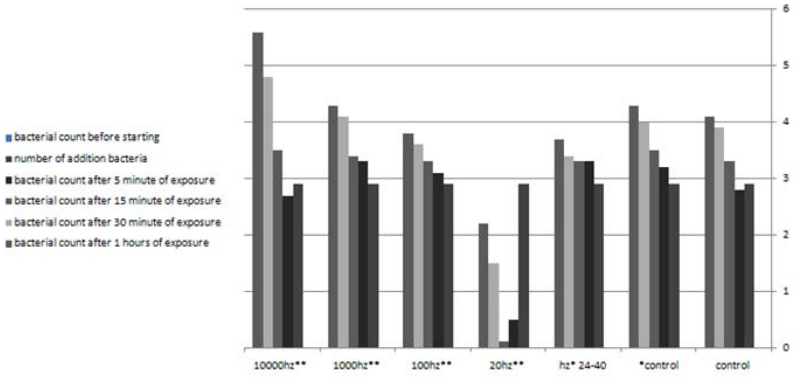

Fig. 1. Comparison of *Aeromonas* count in the water of EMFs treated and control aquaria

Table 3. Total psychrotrophic count in the fish-flesh of Nile tilapia in control and electromagnetic treated group (20hz*) during ice-storage for 7 days

Period/(h)	Control group			Electromagnetic treated groups		
	Min.	Max.	Mean X 10^3	Min.	Max.	Mean X 10^3
0 time	$1.7x10^2$	$6.11x10^2$	$00.43^{Ad} \pm 0.10$	$0.7 x10^2$	$1.3x10^2$	$00.11^{Bd} \pm 0.13$
24	$8.9x10^2$	$10.8x10^2$	$01.92^{Ac} \pm 1.19$	$2.2x10^2$	$4.5x10^2$	$00.68^{Bc} \pm 0.53$
72	$16.5x10^3$	$18.2x10^3$	$17.90^{Ab} \pm 0.20$	$5.2x10^3$	$6.7x10^3$	$6.10^{Bb} \pm 0.43$
120	$14.8x10^3$	$29.0x10^3$	$22.26^{Ab} \pm 4.24$	$11.2x10^3$	$16.0x10^3$	$13.56^{Bb} \pm 0.36$
168	$11.0x10^4$	$16.3x10^4$	$140.10^{Aa} \pm 0.66$	$8.7x10^4$	$11.8x10^4$	$97.93^{Ba} \pm 0.39$

Capital letter = comparison among treatment; Small letter = comparison among time within same treatment;
* :alternative electromagnetic waves.

aminoglycosides, carbapenems, chloramphenicol, and tetracyclines. High resistance of *Aeromonas hydrophila* isolates against most antimicrobials may be explained as; Freshwater streams are usually receptors of many industrial, domestic and agricultural wastes, which could contain antimicrobial agents and antimicrobial-resistant bacteria. Consequently, the freshwater indigenous flora may become a reservoir for antimicrobial genes and the reuse of these waters for humans and animals may contribute to the limitation of antimicrobial's efficiency (Igbinosa *et al*., 2013). The resistance of the strains to amoxicillin, carbenicillin , enrofloxacin, erythromycin , gentamicin , kanamycin, streptomycin , tetracycline were agreed with (Lukkana *et al*., 2012). Susceptibility of *Aeromonas hydrophila* strains in the present study differs among different sources, this may be due to variation of the antibiotic resistance frequencies and profile according to the source of the strains(Ko *et al*., 1996). The problem of antimicrobial resistance is of grave concern. The emergence of multidrug-resistant (MDR) strains and the possibility of transfer of this multidrug resistance to other bacteria have raised the grim specter of bacterial pathogens that cannot be treated by currently known antimicrobials and the reemergence of diseases that can cause large-scale global pandemics. The rise in incidence of MDR bacteria has been attributed to the indiscriminate use of antimicrobials in animal culture and in medicine(Del Castillo *et al*., 2013; Aly, 2013). *Aeromonas hydrophila* from farm-raised Nile Tilapia serve as a reservoir for antimicrobial resistance determinants (Lukkana *et al*., 2011).

The high prevalence of *Aeromonas hydrophila* in tilapia along with their resistance to antimicrobial agents might pose therapeutic problems as well as health risk to consumers. Since antibiotics have been associated with a range of adverse effects, foundation of new strategies becomes necessity. The effects of ELF-EMFs on the biological functions of living organisms represent an emerging area of interest with respect to environmental influences on human health (Segatore *et al*., 2012). Alternative therapies based on electricity or magnetism use verifiable electromagnetic fields, such as pulsed fields, alternating-current, or direct-current fields become an unconventional way to control the bacterial growth. Our results showed that exposure of tilapia aquarium inoculated with anti-microbial-resistant *Aeromonas hydrophila* strains to the effect of alternative low frequency electromagnetic field of electrical waves with 20 hz reduce the bacterial count after 5, 15, 30 and 60 minutes, in comparison to the aquaria exposed to direct low-frequency or alternative high frequency electromagnetic fields under same conditions.

The decrease in the *Aeromonas hydrophila* count in the field of the frequency range 20 hz could be due to :A) Changes in bacterial membrane permeability that could cause biological changes in the organism lead (Comisso *et al*., 2006). B) It might be due to the production of free radicals by bacteria in the electromagnetic field due to the low-frequency fields, while the irrational very low intensity are notable to produce free radicals (Foji *et al*., 2004).Our results agreed with previous reports on the influence of Electromagnetic fields on the bacterial growth (Segatore *et al*., 2012).

Aeromonas hydrophila can grow and produce toxins in refrigerated conditions, indicating that refrigeration cannot be effective enough to control the pathogen (Kirov, 1993). As *Aeromonas hydrophila* are frequently isolated from food due to their pyscrotrophy and existence of pathogen in water. Psychrotrophic count considered to be a better predictor of keeping quality (Downes and Keith 2001),the most important target for keeping overall quality of fish is a decrease in microbial spoilage flora as these cause both decay and safety problems. Psycrhrotrophic count for electromagnetic treated fish were significantly lower than the control group fish at the onset of the experiment and after one, three, five and seven days of ice-storage, Which indicated the positive effect of the electromagnetic fields on the shelf life of the treated fish and so on the keeping quality.

CONCLUSION

This study demonstrated that electromagnetic fields reduce the bacterial growth in both water and fish reared in aquaculture that could improve fish survival and shelf life as well as the keeping quality; this new approach could minimize the use and reduce the risk of antimicrobials use in aquaculture. However, further

investigations about the type, dose and period of electromagnetic field and its safety to exposed fish should be thoroughly implemented before recommending its application in a large scale.

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