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Antibiotic sensitivity and sodium chloride susceptibility patterns of *Flavobacterium columnare* isolated from clinical columnaris in cultured *Clarias gariepinus*

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Antimicrobial resistance is a global concern, especially with the backdrop of the development of possible environmental and public health hazards. *Flavobacterium columnare* has been observed to be multi-drug resistant but highly susceptible to sodium chloride, hence the need for susceptibility profiling. Therapeutic efficacy of commonly used antibiotics and sodium chloride was tested *in-vitro* against four isolates of *F. columnare* using the disc diffusion and the pour plate methods, respectively. Comparative *in-vivo* testing was performed on experimentally infected *Clarias gariepinus* juveniles in 11 groups of 15 fish each. Nine groups were treated with 1.0, 2.0 and 3.0% NaCl for 5, 10 and 30 min each. A tenth group was exposed to 25 mg L^{-1} ciprofloxacin for 1 h by immersion, while the eleventh group was not treated (positive control). Growth inhibition was observed to be highest with ciprofloxacin followed by ofloxacin and tetracycline in that order, and also in all NaCl concentrations. There was no mortality in the infected fish groups treated with 25 mg L^{-1} ciprofloxacin, 1% NaCl, and 2 to 3% NaCl for 5 and 10 min only. However, 46.7 ± 9.4 % mortality observed in 3 % NaCl treatment for 30 min was significantly higher than the 23.3 ± 4.6% observed in 2 % NaCl for 30 min, but not significantly different from the positive control with 36.7±4.7 % mortality. Since short duration sodium chloride bath was found to be effective in the control of columnaris disease, 1% salt disinfection of fry and fingerling stock for 30 min could be incorporated into routine management in catfish hatchery, without the fear of environmental or public health hazards.

Key words: Antibiogram, columnaris disease, sodium chloride, susceptibility.

INTRODUCTION

Flavobacterium columnare has been observed by various researchers (Bernardet and Grimont 1989; Hawke and Thune, 1992) to have developed resistance to many antibiotics, the basis for which its isolation is done on medium made selective with the incorporation of antibiotics to which it is resistant such as neomycin,

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polymyxin B, and tobramycin (Hawke and Thune, 1992; Starliper, 2003). Therefore, where it is desirable to use antibiotics in the treatment of a disease, conducting antibiogram then becomes the rule rather than the exception. Blind use of antibiotics is not only wasteful but also constitutes potential environmental and public health hazard since 75% of the antibiotics still finds its way back to the environment and can further exacerbate the already worsening problems of antibiotic-resistant pathogens.

Common salt (NaCl) often referred to as the 'aspirin of aquaculture' is recommended in bath treatment of fish against some fish pathogens (Bernardet, 1989; Swann and Fitzgerald, 1991; Suomalainen et al*.,* 2005; Noga, 2010). However, the use of NaCl in freshwater, stenohaline fish species will require standardization to ensure effective elimination of pathogens without serious adverse effects on the fish. This study evaluates antibiotic sensitivity profile of isolated *F. columnare*, and validates the efficacy of common salt as a topical disinfectant for the control of *F. columnare* infection.

MATERIALS AND METHODS

Antibiotics sensitivity tests

Antibiotics sensitivity tests were carried out on four isolates of *Flavobacterium columnare* cultured from different clinical cases of columnaris disease. The isolates have been characterized in an earlier study, using species-specific primers (Oladosu et al., 2018). Colonies of 24 h pure culture of the isolates were inoculated into an aliquot of 5 mL cytophaga broth and incubated for 24 h at 25°C. Using a 10 mL automatic pipette and sterile pipette tips, 0.1 mL from each broth culture was spread onto different, newly prepared cytophaga agar plates, and stripped all over the plates to wet the entire surface. The plate for each isolate was properly identified using indelible marker. One antibiotic disc (for Gram-negative organisms) was then dispensed onto the surface of the plates, using sterile pointed forceps to ensure effective contact of the discs with the medium. The plates were then incubated at 25°C for 24 h. The diameter of the visible zone of growth inhibition around the various discs was then measured to determine whether the isolate was sensitive or resistant to which antibiotics. The antibiotics tested include gentamycin, ofloxacin, amoxicillin, ciprofloxacin, tetracycline, augmentin, chloramphenicol, nitrofurantoin, perfloxacin, and cotrimoxazole.

Salinity assay

Analytical grade of sodium chloride (Nacl; BDH - Anala^{R}) was weighed and added to cytophaga broth prior to sterilization by autoclave, to achieve different salinities of 0.5, 1, 2 and 3%. Ten milliliter aliquot of broth with the different salinities was dispensed into 4 bijou bottles for each salinity, and then autoclave at 121°C for 15 min, at 1 atm. Inoculum from each of the four *F columnare* isolates used for the antibiotic sensitivity test was introduced into one bijou bottle each for the different salt concentrations, and incubated with moderate agitation by shaking (150 rpm).

One hundred microliter (100 µL or 0.1 ml) of the inoculated broth was taken from each isolate in the different salinities and dispensed into a sterile petri-dish, to which sterilized cytophaga agar was poured and mixed in a plate-pour method. This was done at different duration of incubation; 5, 10, and 30 min for the different salinities. All the inoculated plates were then incubated at 25^{o}C, and growth examined in 24 and 48 h.

Antibiotic bath and salt disinfection trials

Apparently healthy 180 juveniles (9 weeks old) of *Clarias gariepinus* procured from a commercial fish farm in Ibadan were acclimatized at the rate of 1 fish per 3 L in 30 L water contained in twelve 50 L plastic tanks, for 2 weeks. They were earlier disinfected in 250 ppm formalin for 1 h, and then randomly examined and observed to be free from ectoparasitic infestation, lesions or clinical signs of any disease. Following acclimatization for 2 weeks, they were re-distributed in a population of 30 fish in 30 L of water, at a density 3 times the normal for flow-through culture (300 m^{-3}), and kept in static culture in 6 plastic tanks for 24 h to induce stress.

Thereafter, 81 ml of cytophaga broth culture estimated by McFarland 0.5 standard opacity tube to contain 3.3 x 10^{7} CFU of *F columnare* was introduced into each of the 6 tanks with 30 juvenile *C gariepinus*, containing 30 L of water, to achieve 1.1 x 10^{6} CFU/L. The fish in 5 tanks were exposed to the pathogen for one hour, while those in the 6th tank were not infected (positive control). Fish in all tanks were later re-distributed at 15 fish per tank in 30 L of water in duplicate, and kept in static culture for 48 h before the daily water renewal was restored. Fish were thereafter subjected to treatments by topical bath with 25 mg L^{-1} of ciprofloxacin for 1 h, topical disinfection with sodium chloride at 1, 2 and 3% concentration for 5, 10, and 30 min duration each. The positive control group was not infected while the negative control group was infected but not treated. Two dead fish samples were collected where mortality was observed and inoculum from the skin, the gills and the dorsal kidney were cultured for possible re-isolation of *F. columnare*.

RESULTS

The antibiotic sensitivity pattern observed in this study revealed that all four isolates of *F.columnare* tested were resistant to gentamycin, cotrimoxazole, amoxycilin, augmentin, pefloxacin, ceftriazone, and nitrofurantoin as shown in Table 1. The zone of inhibition to the growth of *F. columnare* observed for ciprofloxacin ranged between 15 to 26 mm with an average of 21.25 mm. This was higher than what was observed for ofloxacin with a range of inhibition zone between 7 and 24 mm, and an average of 13.75 mm. The same trend was observed for tetracycline with a range of inhibition zone of zero to 18 mm and an average of 10.25 mm.

As shown in Table 2, the pattern of susceptibility of *F. columnare* to varied concentrations of sodium chloride indicated that all four isolates tested were resistant to salinity of 0 and 0.5% at all durations of exposure tested. Two isolates were resistant, and the other two susceptible to 1% salinity at 5 and 10 min exposure time, while all four isolates were susceptible to the same salinity at 30 min exposure period. Similarly, all four isolates were susceptible to 2 and 3% salinity at all duration of exposure tested.

As shown in Table 3, no mortality was recorded in infected fish group treated with ciprofloxacin, all duration of exposure (5,10, and 30 mins) for 1% salt bath, as well

Table 1. Size of inhibition zone to *Flavobacterium columnare* growth observed with different antibiotics using disc diffusion method.

Isolates	GEN (10 mg)	COT (25 mg)	OFL (5 mg)	AMX (25 mg)	CPX (10 mg)	TET (30 mg)	PFX (5 mg)	AUG (30 mg)	CRO (30 mg)	NIT (200 mg)
1	0	0	12 mm	0	15 mm	0	0	0	0	0
2	0	0	7 mm	0	18 mm	11 mm	0	0	0	0
3	0	0	24 mm	0	26 mm	18 mm	0	0	0	0
4	0	0	12 mm	0	26 mm	12 mm	0	0	0	0
Mean	0	0	13.75	0	21.25	10.25	0	0	0	0

GENT = Gentamycin; COT= Cotrimoxazole; OFL = Ofloxacin; AMX = Amoxicillin; CPX= Ciprofloxacin; TET = Tetracycline; PFX = Pefloxacin; AUG = Augmentin; CRO = Ceftriazone; NIT= Nitrofurantoin.

as 5 and 10 min duration of exposure for 2 and 3% salt bath. However, mortality was observed even before the expiration of the 30 min exposure period for 3% salt concentration for 30 min exposure. The mortality rate of 46.6% recorded in infected group treated with 3% salt bath for 30 min was significantly higher ($P < 0.05$) than the 23.3% mortality rate recorded for the group treated with 2% salt bath for 30 min. No significant difference ($P < 0.05$) was however observed between these two groups and those that were infected but not treated, where 36.66 % mortality was observed.

Moreover, all water quality parameters tested were observed to be within the acceptable limits, except ammonia levels which though higher than 0.05 mg L^{-1} recommended, can only be toxic when present in the unionized form.

DISCUSSION

The *in-vitro* study on bacterial sensitivity to antibiotics and susceptibility to sodium chloride showed that *F. columnare* was susceptible to ciprofloxacin and sodium chloride concentration of 1% and above. The antibiotic bath and salt disinfection trials reflected the results obtained for the *in-vitro* susceptibility test also, as fish treated with 25 mg L^{-1} of ciprofloxacin and salt concentration of 1% and above were cured of columnaris disease.

The present study demonstrated the efficacy of ciprofloxacin against the four *F. columnare* isolates used, which were observed to be resistant to many frequently used antimicrobials. The bath treatment of infected fish used in this study was also observed to be very effective as no mortality was recorded in fish group treated with the antibiotic as against the 36.6% mortality recorded for the group that was infected but not treated (control). The efficacy of the ciprofloxacin bath treatment lends credence to the observed rapid uptake and tissue distribution of the drug in a pharmacokinetic study with *C. gariepinus* using 25 and 50 mg L^{-1} ciprofloxacin concentration (Oladele et al., 2011).

F. columnare had been reported to be susceptible to salt (NaCl) *in-vitro*, at various concentrations including concentrations above 0.1% (Suomalainen et al., 2005), 0.5% (Shamsudin and Plumb 1996) and 1% (Bernardet 1989). *In-vitro* studies conducted by Bernardet (1989) revealed that the growth of *F columnare* was inhibited at 10 ppt (1%) but not at 5 ppt (0.5%), while Suomalainen et al. (2005) observed that the use of high concentrations of salt and low pH can be a viable treatment option for columnaris disease. These observations formed the basis for the suggestion that salt bath may be an effective control measure against columnaris disease. However, the observations of Suomalainen et al. (2005) seem to prove otherwise. He reported that though 99% of *F. columnare* cells were eliminated in 1 h exposure to 4% salinity, bath treatment did not reflect such efficacy, as 100 % mortality was recorded in experimentally infected fish exposed for 15 min to 4% salinity. The reason adduced for the failure of the bath treatment was that the overlying mucus on the fish shielded the pathogen located on the skin from the disinfectant, based on the buffering capacity of the skin.

In the present study, the mortality recorded in the fish group that was infected but not treated was due to columnaris disease because of the clinical signs observed and the re-isolation of the pathogen from dead fish samples. Mortality rates of 23.3 and 46.6% observed in 30 min exposure to 2 and 3% salinity respectively could be ascribed to lethal NaCl concentrations at prolonged exposure. The absence of clinical signs, observation of signs of stress during bath treatment and commencement of mortality even before the end of the short bath treatment are all indicators of this assumption. Furthermore, *F. columnare* was not isolated from dead fish recovered from this treatments. It should be noted that *C. gariepinus* is a freshwater, stenohaline species, and do not survive or grow for a prolonged period in salinities much above 10 ppt (10 g L^{-1} or 1%). The freshwater stenohaline species are known to regulate their plasma ion such that the internal osmotic pressure of their fluids is equivalent to approximately 10 ppt salinity with a range of 2 ppt, depending on tolerance, regulating

Table 2. Susceptibility pattern of *Flavobacterium columnare* isolates to varied concentration of sodium chloride (common salt) *in-vitro*, observed as bacterial colony count.

Isolate	Salinity (%) / exposure time														
	0.0%			0.5%			1.0%			2.0%			3.0%		
	5 min	10 min	30 min	5 min	10 min	30 min	5 min	10 min	30 min	5 min	10 min	30 min	5 min	10 min	30 min
1	TNC	TNC	300	127	TNC	206	TNC	265	0	0	0	0	0	0	0
2	TNC	TNC	TNC	TNC	TNC	TNC	326	TNC	0	0	0	0	0	0	0
3	TNC	TNC	TNC	TNC	TNC	TNC	0	0	0	0	0	0	0	0	0
4	TNC	TNC	TNC	TNC	TNC	502	0	0	0	0	0	0	0	0	0

TNC = too numerous to count; min = minutes; % = percent or gram of salt per 100 mL of water.

Table 3. Percent mortality observed during bath treatment and topical disinfection of fish experimentally infected with *Flavobacterium columnare* using ciprofloxacin and common salt.

Parameter	Not infected	Infected/ untreated	25 mg L^{-1} ciprofloxacin	1% Salt bath			2% salt bath			3% salt bath		
Duration of Exposure			1 h	5 min	10 min	30 min	5 min	10 min	30 min	5 min	10 min	30 min
No stocked	15	15	15	15	15	15	15	15	15	15	15	15
Mean Weight (g)	38.2 ± 1.4	32.1 ± 0.8	39.3 ± 2.1		36.3 ± 1.1			33.6 ± 2.0			36.0 ± 1.4	
Mortality (%) *	0.0^{c}	36.7±4.72^{ab}	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{c}	**23.3±4.6^{b}**	0.0^{c}	0.0^{c}	**46.7±9.4^{a}**
D.O. range (mg/L)	4.3-5.6	4.6-5.0	5.5-5.8	4.9-5.2	5.5-6.1	5.4-5.7	3.8-6.0	4.2-5.8	5.4-5.6	5.4-6.0	3.8-5.2	5.4-5.8
Temp. range (^{o}C)	25.8-27.0	26.0-27.2	25.1-26.4	26.5-26.8	25.2-27.2	25.8-27.1	25.8-26.2	26.6-27.2	25.4-25.9	25.6-26.6	25.6-25.8	25.1-26.0
pH range	8.2-8.5	7.6-8.2	8.1-8.4	7.4-8.0	7.6-7.8	8.0-8.3	8.1-8.5	8.1-8.3	8.1-8.3	7.8-8.2	8.3-8.5	8.0-8.4
NH_3 range	0.1-0.2	0.1-0.2	0.2	0.2	0.2	0.1-0.2	0.1- 0.2	0.2	0.2	0.1-0.2	0.1-0.2	0.2

*Values are means of two replicates, and values with different superscripts are significantly different ($P < 0.05$).

capacity, and environmental salinity (Brett, 1979). Oladosu et al. (1999) observed the calculated median lethal salinity (MLS – 96) of 7.8 ppt (0.78%) for fingerlings (wt = 1.33 g) of *C. gariepinus*, though ontogenetic variation in salinity tolerance, characteristic of freshwater stenohaline species, was observed as there was increase in MLS – 96 from fertilized eggs to fingerlings.

From the aforesaid, it could be safely inferred that short duration salt bath not beyond 10 min can be used for the disinfection of *C. gariepinus* at salinities of 1 to 3%, to effectively control columnaris disease and concurrent parasitic infection.

Conclusion

The *F. columnare* isolates used in this study are multi-drug resistant, indicating antibiotics mis-use in the aquaculture industry in Nigeria, and suggestive of the need for antibiogram prior to antibiotic treatment of fish. Furthermore, method of disposal of medicated water used in bath treatment of fish should take environmental protection into consideration. Also, since short duration sodium chloride bath was found to be effective in the control of columnaris disease, salt disinfection of fry and fingerling stock could be incorporated into routine management in catfish hatchery.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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