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Virulence of *Pseudomonas* and *Aeromonas* bacteria recovered from *Oreochromis niloticus* (Perege) from Mtera hydropower Dam; Tanzania

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ABSTRACT

The significance of Aeromonas and Pseudomonas bacteria in association with disease outbreaks in feral and aquaculture fish production is of paramount important. Seven isolates of Aeromonas hydrophila (4), A. veronii (2) and Pseudomonas aeruginosa (1) recovered from normal and ulcerative affected Tilapia species in Mtera Dam were examined for virulence. Invitro experiment was conducted in 10 disinfected 20L glass aquaria filled with chlorine free water. 200 healthy Oreochromis niloticus (50–100gm) were used in which 20 fish were stocked in each aquarium. Two aquaria stocked with 10 fish each were used as control. The fish were acclimatized for two weeks prior to the infection experiment. Each fish except the control were intramuscularly injected with 0.1 ml of the experimental bacteria (concentration, 2.4×10^8 CFU/ml) using 21/gauge sterile needle. The infected fish were observed for 14 days. The injected bacteria were then recovered from the experimental fish and subjected to morphological, biochemical and antibiotic susceptibility tests. Results showed that; 112 out of 180 infected fish developed clinical abnormalities such as skin darkness, scales detachment, blindness and large irregular hemorrhages on the body surface, fin necrosis, exophthalmia and eye cataract/trachoma within four days and mortality rate of 95%. The recovered strains were motile, gram- negative, and were resistant to Ampicillin, Streptomycin, Amoxyllin and Novobiocin. This study concluded that Aeromonas and Pseudomonas species are responsible for the ulcerative disease outbreaks in Mtera Dam. However, the study is not conclusive as to whether the same bacteria are responsible for development of eye cataract and blindness to the infected fish.

Key words: Pseudomonads, Aeromonads, Virulence, Mtera hydropower Dam.

INTRODUCTION

Among the etiological agents of bacterial fish diseases *Pseudomonas* and *Aeromonas* are considered one of the most important fish pathogens. These microorganisms are responsible for ulcer type diseases including ulcerative syndrome, bacteria haemorrhagic septicaemia, tail and fin rot, bacteria gill rot and dropsy [1]. These bacterial have been reported to cause septicemia in *Oreochromis niloticus* in Egypt that was more prevalent during winter period [2]

Bacterial pathogens have numerous ways to cause diseases to their hosts. Getting contact to the host and attaching to the host tissues are the most important steps in initiating infections. Virulent bacteria excrete tissue degrading enzymes and toxins to escape the immune defence of the host. Cell surface structure functioning as adhesion factors or having some other roles in the infection process as well as extracellular products have been studied widely in bacterial fish pathogens [1]. Several virulence factors have also been described for bacterial fish pathogens, for instance, capsular material or lipopolysaccharides are related to virulence in *Vibrio* and *Aeromonas hydrophila* [3,

4]. Virulence mechanisms of pathogenic bacteria are important to understand because therein lies the nature of the host–pathogen interaction and knowledge of this allows progress to be made in developing control measures.

Virulence of different Aeromonads and Pseudomonads bacterial isolates have been studied elsewhere in diseased fish in cultured and capture fisheries [5]. In addition, a review of the pathogenicity of gram negative bacterial infections in warm water fishes was reported by [6]. In Egypt, pathogenicity experiment reported *Aeromonas hydrophila* to cause up to 100% mortality in experimental fish (*Oreochromis niloticus)* within 48h. The challenged fish showed extensive skin hemorrhages, fin rot and ulceration. Pathogenicity attributes are present in a high percentage of waterborne strains but their virulence for fish is lower than that displayed by strains isolated from fish [1]. Studies on virulence of bacterial pathogens of fish is essential for the development of new immunoprophylactic and chemotherapeutic reagents to fight bacterial infections, since the development of antibiotic resistance by bacteria has led to diseases becoming one of the major problems in the fisheries sector.

Limited studies are present in Tanzania regarding fish diseases in general. The prevalence of pathogenic bacteria (Pseudomonads and Aeromonads) infections in fish and their virulence in both feral and culture conditions is yet to be investigated in spite of pathogenic bacteria being responsible for heavy economic losses caused by both high mortality and deterioration of product quality [7, 8]. To avoid losses that might arise due to emergent fish infections; investigation of pathogenic bacteria in fish and their virulence encounter a routine significance. In this study, investigation on the virulence of *Aeromonas* and *Pseudomonas* bacteria isolated from *Oreochromis niloticus* (Perege) was the main objective.

MATERIALS AND METHODS

Seven isolates of *Aeromonas hydrophila* (4), *A. veronii* (2) and *Pseudomonas aeruginosa* (1) from normal and diseased feral and cultured fish were tested for virulence to *Oreochromis niloticus* following the method by [9]. Prior to injection; 200 healthy *Oreochromis niloticus* weighing 10 – 100gm were obtained from the practical ponds in the Tanzania National Museum, transferred and maintained in ten glass aquaria supplied with de-chlorinated tap water with aeration and allowed to acclimatize for 2 weeks. The experimental fish were fed twice a day with commercial pellet feed. The physical chemical characteristics of the experimental water was maintained throughout the experimental period.

To each experimental fish, 0.1 ml of 2.4×10^8 CFU/ml of the isolated bacterial suspension was intramuscularly injected using 21 gauge sterile needle. Ten fish were injected with Phosphate Buffered Saline (PBS; pH 7.2) [control (1)] using the same procedure. Another 10 fish were held untreated [control (2)]. The observation time was 14 days**.** The virulence of the strains was categorized on the basis of development of clinical signs and percentage mortalities. 90% to 100% mortality within 24h as highly virulent; Over 50% mortality and lesions within 24-48h as moderately virulent and over 50% mortality with hemorrhagic lesions after 48h but within a specified time period of 120h as avirulent. The bacteria strains were re-isolated from the dead and from fish with clinical conditions and examined.

Anti-bacterial activity of different drugs against *Aeromonas* and *Pseudomonas* species isolated from fish.
Thirteen antimicrobial drugs were evaluated for effectiveness against *Aeromonas* and *pseudomonas* strains using disc diffusion technique [10]. Stock cultures of the virulent strains were grown in nutrient broth for 24h at 28^{o}C. After centrifugation at 5000g for 30 min at 4^{o}C, bacteria were re-suspended in sterile Phosphate Buffered Saline (PBS) and diluted to a turbidity equivalent to a McFarland No. 0.5 standard solution (0.5 ml $BaSO_4$ + 99.5 ml 0.36 N HCl).

0.1ml of the bacterial suspension was spread onto Mueller–Hinton agar (Difco) and chemotherapeutic agent discs were then added and the preparation incubated at 28^{o}C for 24 h (Bauer et al. 1966). The chemotherapeutic agent used included three cell wall synthesis inhibitors (Ampicillin, 10 µg, Amoxicillin 20 µg and penicillin G, 10 IU); seven protein synthesis inhibitors (Chloramphenicol, 30 µg; erythromycin, 15 µg; gentamicin, 10 µg; kanamycin, 30 µg; neomycin, 30 µg; streptomycin, 10 µg; tetracycline, 30 µg) and three nucleic acid synthesis inhibitors (ciprofloxacin, 5 µg; Novobiocin, 5µg and Trimethoprim-sulfamethoxazole, 25 µg) Table 2. Characterization of strains as resistant, intermediate, or sensitive was based on the size of the inhibition zones around each disc according to standards by [11].

RESULTS

Virulence for fish
Of the seven bacterial strains used during the infection trial; strains 1, 2,3,4 and 7 were highly virulent to the experimental fish in which they caused mortality ranging between 70% to 95% within 48 h (Table 1) with the dose

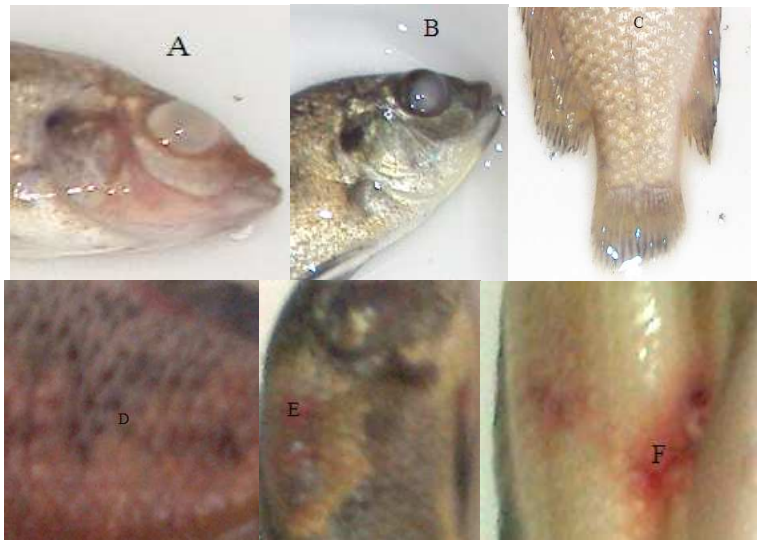
of bacteria at LD_{50} value of 2.4 $X10^8$ CFU ml^{-1}, while strains 5 and 6 were classified as avirulent according to the degree of virulence described by [12].

Table 1. Virulence results for Oreochromis niloticus of Aeromonas and Pseudomonas species after intramuscular injections

Isolate No.	Number of fish/ isolate	Fish mortality / isolate/ hrs.	% Virulence
1 (250S) *Aeromonas veronii*	20	19/24	95
2 (120K) *Pseudomonas aeruginosa*	20	18/24	90
7 (8.7 Intest) *A. hydrophyla*	20	15/48	75
4 (80G) *A. veronii*	20	16/48	80
3 (80T) *A. hydrophila subsp. dhakensis*	20	17/48	85
5 (126K *A. hydrophyla*	20	14/96	70
6 (600K) *A.* species	20	13/120	65
Control I	10	0/10	0
Control II	10	0/10	0

Clinical Observation

The results for the virulence test are summarized in Table (1). Out of 200 experimental fish treated, 112 fish (56%) showed clinical abnormalities including skin color darkness, detachment of the scales, large irregular hemorrhages on the body surface, shallow to deep necrotizing ulcers on the skin, fin necrosis, inflamed vent, exophthalmia, Blindness and eye cataract/trachoma, (Plates A-F). No clinical abnormalities or death confirmed in the control fish.



Plates A, B and C- The clinical abnormalities after injection with *Pseudomonas* species; D, E and F the after injection with *Aeromonas* species

Table. 2 Summary of Antibiotic susceptibility patterns of highly virulent *Aeromonas* and *Pseudomonas* species

Antibiotic Disc	Response of bacterial strains to different antibiotics				
	Strain 1 (250 Skin)	Strain 4 (120 Kidney)	Strain7 (8.7 Intestine)	Strain 2 (80Gill)	Strain 3 (80Tissue)
Penicillin G	R	S	R	R	R
Kanamycin	S	R	S	S	S
Gentamicin	S	R	S	S	S
Chloramphenicol	S	S	S	S	S
Ampicillin	R	R	R	R	R
Streptomycin	R	R	R	R	R
Amikacin	S	S	S	I	S
Amoxicillin	R	R	I	R	R
Trimethoprimsulfamethoxazole	R	S	S	R	S
Erythromycin	R	R	S	I	S
Tetracycline	R	R	S	R	R
Ciprofloxacin	S	S	S	S	S
Novobiocin	R	R	R	R	R
Neomycin	S	R	S	S	S
Summary S/R	6/8	5/9	9/5	5/9	8/6

Key: S- Susceptible; R- Resistant; I- Intermediate based on the size of the inhibition zone around the disc as described in [11]

Anti-microbial sensitivity test

The results of antimicrobial tests revealed that most of the tested drugs (51.43%) were effective against the re-isolated strains. This provides a wide range of drugs for fish farmers to obtain treatment to their fish in case of bacterial disease outbreaks that is associated with *Aeromonas* or *Pseudomonas.* About 48.6 % of the drugs were not effective to the re-isolated strains.

DISCUSSION

Although the experiment involved seven isolates, only five were found to be virulent; the other two isolates; (*A.* species and *A. hydrophila* from the kidney) were either avirulent or weakly virulent according to the degree of virulence described by [12]. Using morphological, physiological and biochemical characteristics; strain 4 was identified as *P. Aeruginosa*. *P. aeruginosa* is reported to be the only gram -negative bacillus capable of producing distinctive water soluble pigment, pyocyanin [13]; strains 1 and 2 were confirmed as *Aeromonas veronii*, whereas strains 3 and 7 were *Aeromonas hydrophila* [14]. Both strains were considered to be virulent as they caused clinical abnormalities with mortality above 70% within 48h. The isolated bacterial species are reported to cause haemorrhagic septicaemia and ulcerative diseases in finfish in Mtera hydropower Dam and shellfish elsewhere [1, 5, 14-17].

From the bacterial challenge experiments, a dose of bacteria at LD_{50} value of 2.4 $X10^8$ CFU ml^{-1} of strains *A.veronii* (2), *Pseudomonas aeruginosa* (1), *A. hydrophila subsp dhakensis* (1) and *A. hydrophila* (3) were able to cause 75% to 95% mortalities within two days while strains of *A. hdrophyla* and *Aeromonas* species from the Kidney caused up to 70% mortalities after two days but within the specified period of three days. Although the physical chemical parameters were monitored during the entire experimental period the degree of virulence was different among the strains. The reasons for this variation are not clear. In line with the degree of virulence stated by [12], *A. veronii* from the skin and *Pseudomonas aeruginosa* from the Kidney were highly virulent whereas *A. hydrophila subsp dhakensis* from the muscle tissue, *A.veronii* from the kidney and *A. hydrophila* from the intestine were moderately virulent. Previous study on virulence conducted in Bangladesh [5] revealed that *Pseudomonas* and *Aeromonas* caused high mortality up to 50% in the experimental fish using intramuscular injection method at a bacterial challenge dose of 3-5$x10^6$ CFU/ml. Another study in the Philippines [18] reported *Aeromonas hydrophila* causing cumulative mortality of 50% in the experimental fish. This is lower level of virulence compared to the present study. In summary antibiotic susceptibility assays revealed that 51.43% of the bacterial strains tested were susceptible to the chemotherapeutic agents used although there was individual cases were the strains were almost resistant to more than 80 to 90 % of the tested drugs (Table 2). This result suggests that antibiotics could be employed to prevent outbreaks of diseases particularly in confined environments but not always can eliminate most of strains as some virulent bacteria have developed resistance to most of the chemotherapeutic agents. Efforts are needed to control the disease from occurring rather than treating the disease which is most of the time risky and expensive. In this study, *Aeromonas hydrophila* was sensitive to Erythromycin, Neomycin, chloramphenicol, Trimethoprim-sulfamethoxazole and Kanamycin. In contrast, *A. hydrophila* strains isolated from fish in Malaysia were reported to be resistance to most of the drugs used in this experiment [19]. The observed differences in the frequency of resistance might be due to the source of *Aeromonas* isolates and the frequency and type of antimicrobial agents prescribed for the treatment of *Aeromonas* infections in different geographic areas [19]. *Aeromonas veronii* have recently been reported to cause acute death of Channel Catfish *(Ictaluruslunetas*) in China with severe ascites, extensive hemorrhage on the body surface and organs [20]. Similar clinical characteristics were observed in this experiment. *A. hydrophila* is described as the dominant infectious agent of 'fish-bacterial-septicemia' in freshwater cultured finfish in China [21] *Aeromonas hydrophila* is also associated with EUS, which is a major problem in Southeast Asia, [22].

Fish diseases caused by *Aeromonas* and *Pseudomonas* have been considered to be the major bacterial problems facing the aquaculture development causing mass mortalities, reduced reduction and low quality of aquatic organisms. Both Aeromonas spp. (*A. hydrophila, A. sobria and A. caviae*) and Pseudomonas spp. (*P.fluorescens, P. putida and P. aeruginosa*) were incorporated in severe outbreaks among *O. niloticus* in fish hatcheries [2].

The findings from this study are in agreement with several studies conducted elsewhere in which *Pseudomonas* and *Aeromonas* were studied for virulence [1, 2, 6, 18, 23-25] . The findings from this experiment can be used to simulate the actual situation happening in the wildness during the disease outbreaks. It is the first extensive study in fish diseases in Tanzania where *Aeromonas* and *Pseudomonas* are reported as the causative agent of fish infections. The findings also indicated some of the infected fish to develop blindness of the eyes and trachoma; although there are several reports on fish blindness due bacterial infections; no specific bacterial have been mentioned to cause the blindness. In this experiment the re-isolated bacteria from the fishes that developed blindness were *pseudomonas*, however, the study was not conclusive of whether pseudomonas was the real causative agent of the problem or not.

Clinical abnormalities developed by the experimental fish have been of important in fisheries management; for example, fin erosion has become a concern in fisheries management because of aesthetic and fish survival issues [6]. The erosion of the fins in fish is reported to be caused by several factors including abrasion with rough surfaces, fin damage from aggressive encounters between fish, nutritional deficiencies, and bacterial infection. However from this study the clinical observations after the bacterial treatment are to a larger extent resulting from the bacterial infections as other survival conditions were monitored.

CONCLUSION

The present study has provided an important understanding on some of the most pathogenic bacterial strains and their virulence for potential bacterial fish pathogens in Tanzania. The information will help in controlling and treating the incidents of bacterial infections in aquaculture ventures as well as in capture fisheries. This knowledge will be of significant to fish farmers in maintenance of fish health for improvement of fish productions and ultimately reflects the economy of the farmers and nation as a whole.

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