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Occurrence and antibiotic sensitivity profile of *Salmonella* species isolated from plastic fish ponds

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ABSTRACT Bacterial pathogens are a major menace in the aquaculture industry therefore; the success of any aquaculture system is directly proportional to the ability of the fish farmer to successfully manage the health of the fish. This study was carried out to evaluate the antibiotic sensitivity profile of *Salmonella* species isolated from plastic fish ponds in Port Harcourt using standard techniques. The samples used for the study were borehole water, borehole water and fish feed and borehole water and feed and fish excreta obtained from the ponds. *Salmonella* Species were isolated using the spread plate technique and identified using biochemical methods. Antibiotic susceptibility pattern of the isolates was determined using the disk diffusion technique. A total of 628 *Salmonella* species were isolated from all the samples of the fish ponds. Sensitivity result showed that *Salmonella* species exhibited varying sensitivities to the same antibiotic being sensitive, resistant or intermediate. The susceptibility of isolates to test antibiotics were Tarivid (100%), susceptible to Gentamicin and Peflacine (67% each), Ciproflox and Septrin (56% each), Augmentin (44%), and Ampicillin (22%). The resistance of isolates was Ampicillin (78%), Ceporex (56%), Augmentin (44%), Nalidixic acid and Septrin (33% each) and to Peflacine and Ciproflox (11% each). Almost all the *Salmonella* isolated from the three samples exhibited multiple antibiotic resistance. From the findings of this research, it is imperative that the borehole water, fish feed and indeed all inputs in the aquaculture process be closely monitored as any of them could be a source of *Salmonella* contamination of the cultured fish. Equally, the indiscriminate and uncontrolled use of antibiotics in the aquaculture process should be closely regulated.

Keywords: Fish ponds. Borehole water, *Salmonella*, antibiotic resistance

Introduction

Fish is an important aspect of the human diet; it serves as a cheap source of protein and it also a rich source of minerals and nutrients for the sustenance of man. (FAO, 2002). The aquaculture industry, has been said to provide about half of all the animal protein which is needed by the human population and it has also provided sustenance to individuals in terms of employment (Khainar *et al*., 2013; Bostock *et al*., 2010) Common species of catfish farmed in the African catfish aquaculture industry are the *Heterobranchus* species, *Dutch clarias* and the *Clarias gariepinus*. Anetekhai *et al.,* (2004)

The characteristics of the African catfish that make it a ready choice for commercial rearing according to Anyanwu and Chah (2016) and Anetekhai *et al.,*

(2004) are its resilience, ability to withstand the tropical environment, its ability to withstand culture with other fresh water species, tolerance to high stocking densities, high fecundity rate, ability to be transported either alive or smoked to where it is sold, high nutritional value and weight gain.

However, in spite of these glowing attributes the success of any aquaculture system is directly proportional to the ability of the fish farmer to successfully manage the health of the fish, reason being that bacterial pathogens are a major menace in the aquaculture industry (Anyanwu and Chah, 2016).

The sources of these pathogens are many and varied and they include according to Nagham *et al*., (2015) the practice of using animal manure to augment conventional fish feed especially in an integrated farming structure. Other sources of these pathogens are poor water quality, poor handling of fish, infrequent water change, uneaten floating feed and high stocking densities (Njoku *et al*., 2015a).

To curb the menace of bacterial pathogenic invasion, the fish farmer employs a myriad of antibacterial agents to inhibit and possibly wipe out the invading bacterial species to avert economic losses to his fish farm.

Sadly, a greater proportion of the countries where the African catfish is farmed are developing and less industrialized nations where the regulation of antibiotic use is nonexistent and this has in turn contributed greatly to the development of antibacterial resistance (Cabello *et. al.,* 2013)

Also, the widespread use and misuse of antibiotics in treating infections and its use in veterinary, agriculture and aquaculture industries have resulted in the widespread incidence of antibiotic resistant bacteria in the environment (Hongyue and Song, 2008).

In addition, there exists the potential of transfer of resistant bacteria and resistance genes from the aquaculture environment when fish which are loaded with antibiotic resistance bacteria are consumed (Omojowo and Omojosola 2013). The continued, indiscriminate and widespread use of antibiotics exerts a selective pressure that plays a key role in the development of resistance (Obire *et al*., 2009).

The aim of this study is therefore to examine and determine the occurrence and the antibiotic sensitivity profile of *Salmonella* species obtained from three samples from each of the three plastic ponds covered in the scope of this research.

Materials and Methods

Study area

The study was conducted in the Port Harcourt metropolis of Rivers State located within longitude 7.0498º E and latitude 4.8156º N with a land mass of 360km squared. The vegetation of the area reflects a tropical wet climate with lengthy and heavy rainfall and a very short dry season. The dry season which climatically influences many cities in West Africa is less pronounced in Port Harcourt. Informing the choice of the study area is the fact that the plastic ponds are in close proximity to three popular markets namely the Mile one market, Fimie market and the Creek road market respectively. These markets serve as possible sales outlets for the fish harvested from these ponds.

Collection of Samples

Water samples were obtained from three plastic fish ponds located at D-Line, Ozuboko and the UPE Sand fill Borikiri areas of Port Harcourt. These areas of Port Harcourt are densely populated areas and due to the unavailability of land to carry out the earthen pond system, plastic ponds are used instead Three samples namely the raw water from borehole which is pumped into the plastic pond, the fish pond water after fish feed has been added to it and the fish pond water containing fish feed and fish excreta were collected from each fish pond. These three water samples were selected for investigation in each of the ponds so as to ascertain which of them is a possible source of *Salmonella* pathogenic contamination in the aquaculture process. Microbial count samples were collected in sterile screw capped containers which were rinsed with the sample before collection. The collected samples were stored in an ice cooler and transported to the laboratory for analysis within 2 hours of collection.

Isolation of Salmonella species

Water sample (0.1ml) of each pond was inoculated directly onto *Salmonella-Shigella agar* (SSA) (Oxiod limited, Wade Road, Basingstoke Hampshire United Kingdom). Pure colonies of *Salmonella* were picked, sub cultured and stored for future examination.

Characterization and Identification of Isolates

The pure isolates obtained were characterized morphologically, microscopically and biochemically using a number of biochemical tests (Holt *et al*., 1994).

Antibiotic resistance testing

The antibiotic sensitivity pattern of the bacterial isolates obtained from the fish ponds was determined according to the Kirby Bauer method (Bauer *et al*., 1979). The broth culture of each isolate was standardized using 0.5 McFarland standard. The test organism was inoculated on Sterile Mueller Hinton agar and the gram negative antibiotic disc was placed on the inoculated medium and the inoculated plates incubated at 37^0C for 16-18 hours. The gram-negative disc was impregnated with the following antibiotics, Tarivid (10µg), Peflacine (10µg), Ciprofloxacin (10µg), Augmentin (30µg), Gentamicin (10µg), and Streptomycin (30µg). Septrin (30µg), Ceporex (10µg), Nalidixic acid (30µg), Ampicillin (30µg). Results were recorded by measuring the zones of inhibition and comparing them with (WHO/LAB/87. 1; Reynolds (2005) and classified as resistant, intermediate and susceptible.

MAR Indexing of Isolates

MAR index = a÷b where "a" is the number of antibiotics to which the particular isolate was resistant while "b" is the number of antibiotics to which it was exposed. MAR index values which are above 0.2 indicates multiple antibiotic resistance and are considered to have their origin from sources which are considered as high-risk such as poultry droppings and livestock droppings where antibiotics are frequently used. MAR values of less than or equal to 0.2 indicate a strain that originated from animals in which antibiotics are seldom used (Lee *et al.*, 2016).

Results

The result of the counts of *Salmonella* species recorded for the various samples of the fish ponds showed that, counts of *Salmonella* species in sample A (borehole water) ranged from 2.0 × 10^3 CFU/ml to 1.0 × 10^3 CFU/ml for the D-Line fish pond, no growth in the Ozuboko fish pond borehole water and ranged from 1.0 × 10^2 to 4.0 × 10^2 in the UPE Sandfill fish pond with a mean count of 1.5 ×10^3 CFU/ml; 0; and 2.5 × 10^2 CFU/ml respectively.

The counts of *Salmonella* species in sample B (borehole water + fish feed) ranged from 1.0 ×10^3 CFU/ml to 7.0 × 10^3 CFU/ml for D-Line fish pond, from 1.6 × 10^4 CFU/ml to 1.5 × 10^4 CFU/ml in Ozuboko fish pond and from 5.2 × 10^3 CFU/ml to 9.0 × 10^3 CFU/ml in the UPE Sandfill fish pond with a mean count of 4.0 ×10^3 CFU/ml; 1.55 × 10^4 CFU/ml; and 7.1 × 10^3 CFU/ml respectively.

The counts of *Salmonella* species in sample C (Borehole water + fish feed + fish excreta) ranged from 2.0 × 10^4 CFU/ml to 7.4 ×10^4 CFU/ml for D-Line fish pond, from 1.5 × 10^4 CFU/ml to 1.8 × 10^4 CFU/ml in Ozuboko fish pond and from 1.5 × 10^4 CFU/ml to 1.29 × 10^4 CFU/ml in the UPE Sandfill fish pond borehole water with a mean count of 4.7 × 10^4 CFU/ml; 1.65 × 10^4 CFU/ml; and 1.395 × 10^4 CFU/ml respectively.

The occurrence (%) of *Salmonella* species and error bars with standard error of the various samples of the fish ponds is shown in Figure 1.

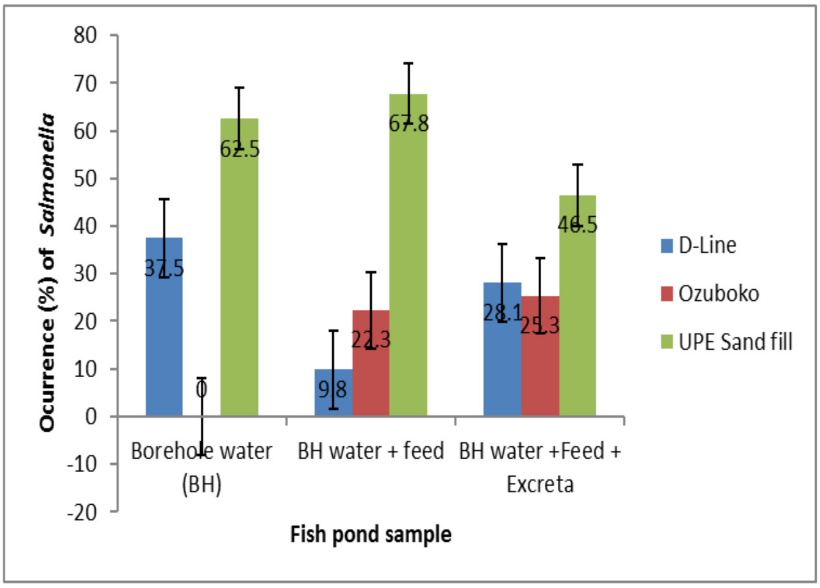

Fig. 1: Occurrence (%) of *Salmonella* species in samples of the fish ponds

The antibiotic sensitivity profile of *Salmonella* species of the fish ponds is shown in Figure 2.

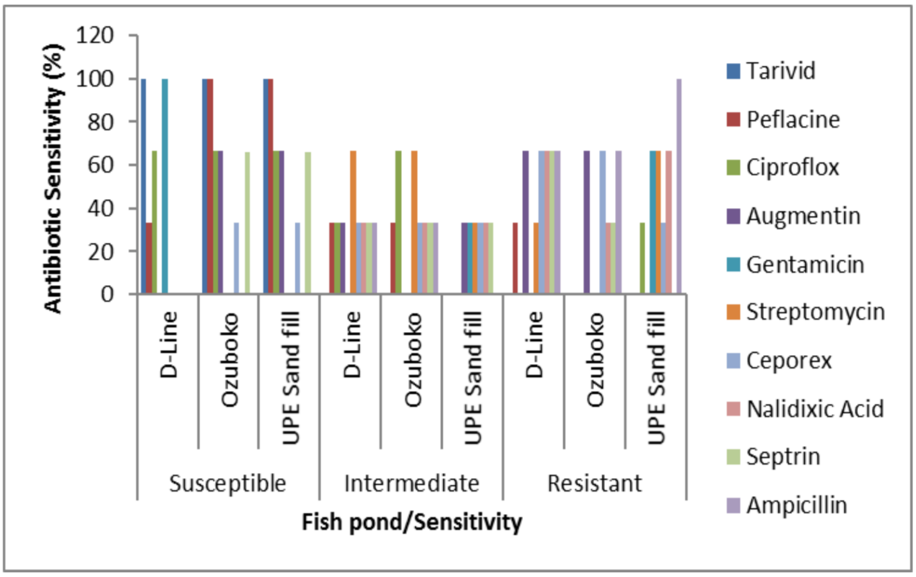

Fig 2: Antibiotic sensitivity profile of *Salmonella* species of the fish ponds

The antibiotic sensitivity profile showed that the susceptibility of the *Salmonella* isolates from `the three fish ponds to test antibiotics were Tarivid (100%), susceptible to Gentamicin and Peflacine (67% each), Ciproflox and Septrin (56% each), Augmentin (44%), and Ampicillin (22%). The resistance of isolates was Ampicillin (78%), Ceporex (56%), Augmentin (44%), Nalidixic acid and Septrin (33% each) and to Peflacine and Ciproflox (11% each).

The result of the MAR index of *Salmonella* species of the fish pond samples is shown in Table 1 below. The MAR index values for the isolates ranged from 0 to 0.6. The result of the MAR indexing indicates multiple antibiotic resistance in some of the isolates.

Table 1: MAR index of *Salmonella* isolates from the fish pond water samples

Fish Pond	Borehole water (BH)	BH water + feed	BH water +Feed + Excreta
D-Line	0.6	0	0.6
Ozuboko	0	0.4	0.3
UPE Sand fill	0.4	0.4	0.1

Discussion

Salmonella is regarded as a notorious pathogen implicated in the pathology of human gastroenteritis (EFSA and ECDC, 2015), it is a mesophilic organism and does not have water as its natural habitat, however it has been found to possess the ability to survive for extended periods in aquatic environments, surviving up to 15 days in septic tanks, 54 days in water and 119 days in sediment samples (Moore *et al*., 2000). *Salmonella* has also been reported to persist in form of biofilms during periods of nutrient depletion and dryness (Aleksandr *et al*., 2015). It is therefore important to understand the microbial flora associated with the fish culture environment since the aqueous environment of fish is reflected in its microbial flora (Erondu and Anyanwu, 2005). The presence of *Salmonella* in the aquaculture environment can be attributed to hygiene mishaps during the aquaculture process (Li *et al*., 2009). The findings of this study reflect the presence of *Salmonella* species from two out of three of the borehole water samples analyzed from the three fish ponds. Findings from Amagliana *et al.,* (2012) indicate that *Salmonella* can contaminate water sources as a result of poor sanitation and incorrect disposal of human and animal waste.

The practice of disposing refuse into gutters when it rains, a common practice in Port Harcourt could be a contributory factor that accounts for the presence of *Salmonella* in borehole water. In addition, residents around the river banks of Port Harcourt usually empty their domestic waste into the surrounding water, this in addition to connecting the sewer pipes that receives their faecal waste directly into the surrounding rivers. This underhanded and unhygienic practice could be responsible for possible ground water contamination which is in turn drilled by prospective borehole owners and pumped into these fish ponds.

Findings from this study also implicated the feed fed to fish as a possible source of *Salmonella* contamination, as all samples obtained from the three ponds recorded *Salmonella* isolates in numbers that were significantly higher than what was obtained in the borehole water sample. This corroborates the findings of Budiati *et al*., (2013) and Lunestad *et al*., (2007) that attributed the presence of *Salmonella* in the aquaculture process to the fish feed which contain organic materials and introduces a wide variety of microorganisms which is fed to the fish in these ponds.

Salmonella was also found to be present in all the samples marked as sample C (fishpond water in addition to the fish feed and fish excreta). Kumar *et al*., (2009) is of the view that filter feeders accumulate these pathogens in their tissues as a result of filtration in the course of their feeding. It is therefore possible that these pathogens are also excreted into their aqueous environment in the course of their metabolism thus accounting for the higher counts of *Salmonella* species in sample C compared to sample B. Thus, there was an increase in *Salmonella* species from bore hole water, to borehole water plus fish feed to borehole water plus fish feed and fish excreta.

Investigations into the antibiotic sensitivity profile of *Salmonella* species obtained from all the three samples indicate that strains of the same bacteria in this particularly study; *Salmonella* species obtained from the same source exhibit varying degrees of resistance or sensitivity to the same antibiotic. Results indicate that no isolate was 100% resistant to all the tested antibiotics, studies report that the ability of *Salmonella* to form biofilms increases their chances of survival in fresh water environments and also confers on them ability to be less sensitive to antimicrobial agents and disinfectants compared to planktonic bacteria (Janssens *et al*., 2008;

Moretro *et al*., 2009). *Salmonella* isolates demonstrated more resistance to cheaper, commonly used antibiotics such as Ampicillin compared to less known antibiotics such as Tarivid and Peflacine to which isolates were more susceptible as demonstrated in the study. This suggests that the indiscriminate and unprescribed use of antibiotics by individuals and farmers have conferred antibiotic resistance on bacterial isolates. According to Obire and Amadi (2015), inexpensive antibiotics are widely available without prescription from unauthorized patent medicine shops. In addition, the habit of adding antibiotics to herbal preparations is another scourge that may have led to the steady rise in antibiotic resistance. It is common knowledge that Ampiclox® is usually added to the herbal drug 'seven keys' which is ingested orally as a treatment for chicken pox which is a viral infection (Obire *et al*., 2009).

Obire *et al*., (2009) holds the view that poor hygiene, suboptimal sanitary conditions and infection control which are listed as risk factors for antibiotic resistance in bacteria are prevalent in Nigeria and other developing countries. A case in point is Fish pond 3 which operates a system whereby pond water is drained every 48 hours as against daily water change in the other ponds, this has resulted in the accumulation of metabolic waste products in the pond water, in addition to putrefying uneaten or leftover feed which further pollute the water and possibly accounts for the high prevalence of *Salmonella* in its water samples and the 100% resistance recorded for Ampicillin.

Investigations by Njoku *et al*., (2015b) and Omojowo and Omasola (2013) recorded a susceptibility value of 70% and 100% to Ciprofloxacine and Ofloxacine respectively. Omojowo and Omojosola specifically recorded a susceptibility value of 100% by *Salmonella* to Ofloxacin. Consequently, the drug was recommended as the drug of choice in the treatment of infections in the aquaculture industry, however the findings of this more recent research reveal a susceptibility value of 56% to ciprofloxacin, this signals a decline in susceptibility ratings of the drug and a corresponding increase in resistance values, this indicates that sustainable and continued antibiotic susceptibility and resistance surveillance be carried out to monitor the trend and subsequently interpret the findings of the monitoring exercise as it affects the health of individuals with regards to drug prescription.

The result of the MAR indexing of isolates reveals that there exists MAR (multiple antibiotic resistant) isolates across the three ponds. The values obtained, which are 67% higher than 0.2 indicate that the *Salmonella* isolates present in the samples have their origin as human, poultry or cattle waste. This corroborates findings stating that drug resistant *Salmonellae* are introduced in the aquaculture process as a result of human waste which is utilized in the aquaculture sector of developing countries. In addition, the culturing of fish in oxy tetracycline treated ponds is a wide spread practice, this has been discovered to increase levels of antibiotic resistance (Lee *et al*., 2016).

The D-Line fish pond demonstrated MAR index of 0.6 in sample A (borehole water) and Sample C (fish feed plus fish excreta plus borehole water) this suggests that drug resistant *Salmonellae* are present right from the borehole water which is the aqueous source.

Ozuboko fish pond demonstrated MAR values of 0.4 in sample B (borehole water plus fish feed) and a value of 0.3 in its sample C (fish feed plus fish excreta plus borehole water). UPE sand fill fish pond had values of 0.4 in its sample A (borehole water), 0.4 in its sample B (borehole water plus fish feed) and a value of 0.1 in sample C (fish feed plus fish excreta plus borehole water). The MAR values recorded across the three ponds suggests the risk associated with these water samples and especially with the waste water which is routinely discharged into the environment as it could serve as a reservoir for the spread of antibiotic resistant bacteria in the environment.

Conclusion

Salmonella species is clearly a pathogen associated with farmed fish and its aqueous environment. It is worthy of note that it was present right from the borehole water which was pumped into two of the three fish ponds studied. Of greater public health interest is its percentage of sensitivity and resistance to tested antibiotics. It is therefore clear that antibiotic resistance is a global health challenge as organisms are constantly evolving in order to reduce the efficacy of tested antibiotics. The sources of this resistance could be linked to man's indiscriminate use of antibiotics, practice of growing fish in oxy-tetracycline treated ponds, culturing fish in less than sanitary conditions, and unhygienic feed which is fed to fish. The implications of the finding of this study suggests that there is a public health risk associated with consuming fish farmed in some of these ponds considering the pathogenicity of *Salmonella* species, in addition to its increasing level of susceptibility to named antibiotics and multiple antibiotic resistance.

Recommendations

To curb this challenge of antibiotic resistant *Salmonella* species in fish ponds, it is important that regulatory bodies should repeatedly sensitize the public on the ills associated with emptying domestic and faecal waste into open drains and rivers as it could possibly contaminate the water table especially in shallow areas introducing pathogenic organisms into borehole water that is otherwise considered as pure. Public health officers should also carry out monitoring on the efficacy or otherwise of antibiotics to tested organisms so as to guide drug prescription and dosage administration.

In addition, aqua culturists should be properly educated on the optimum conditions and practices that allow for the growth of healthy farmed fish, and also the right drug and dosage to be administered in the event of fish morbidity. Critical controls and HACCP (hazard analysis critical control points) should be inculcated into the aquaculture process from start to finish. The indiscriminate and unwholesome use of antibiotics should also be discouraged and antibiotics should be purchased purely on a prescription basis only. In addition, borehole water should be treated as they could be a possible reservoir of *Salmonella* species, it is also important to enforce the sighting of borehole drills at the recommended WHO distance away from septic tanks in order to avoid possible contamination.

Considering the economic viability of the aquaculture industry, policy makers should consider the possibility of subsidizing the cost of conventional fish feed so as to discourage the practice of feeding fish either with human or animal waste (especially poultry droppings) as it serves as a source of drug resistant *Salmonella.*

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