Efficacy of some synthetic antibiotics on *Streptococcus pneumoniae* and *Proteus mirabilis* isolated from fish tanks

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Abstract

Effects of some synthetic antibiotics on Streptococcus pneumoniae and Proteus mirabilis isolated from cultured Clarias gariepinus, an important food fish raised in a concrete tank was carried out to ascertain their remedies on mortalities of the Clarias gariepinus adult fish. Streptococcus pneumoniae and Proteus mirabilis were isolated from the gill and skin of diseased fish and water samples and identified by standard microbiological methods. The bacteria isolates were tested for sensitivity on ten (10) antibiotics namely Amoxycillin, Gentamicin, Nitrofurantoin, Erythromycin, Augmentin, Tetracycline, Naladixic acid, Cotrimoxazole, Cloxacillin and Ofloxacin. These organisms reacted differently to the different antibiotics that were used - Ofloxacin, Gentamicin and Erythromycin had highest zones of inhibition of 26mm and 26mm, 24mm and 19mm, 16mm and 17mm respectively around Streptococcus pneumoniae and Proteus mirabilis, but Naladixic acid, Augmentin, Cloxacillin and Amoxycillin had no inhibition zones around both organisms. It is therefore recommended that Ofloxacin, Gentamicin and Erythromycin can be used to combat S. pneumoniae and P. mirabilis diseases in fish farms because of their high antibacterial potentials against the test organisms.

Keywords: *Streptococcus pneumoniae, Proteus mirabilis, Clarias gariepinus, Synthetic antibiotics*

Introduction

Fish diseases are one of the major problems on fish farms. Intensification of fish farming in Nigeria has increased the number of disease outbreaks in intensive production systems. Most etiological agents were not yet identified and their morbid processes still not studied. Outbreaks of diseases are associated with depressed oxygen levels, predisposing risk factors include also overcrowding and organic pollution (Kirjusina *et al.*, 2007). In fish farms, outbreaks are typically associated with stress, especially, high temperatures and low oxygen levels. Bacteria are important pathogens for both cultivated and wild fish, and are responsible for serious economic losses. Inglis *et al.* (2001), reported that some bacteria cause

only surface diseases on skin or gill infections, especially flexibacteria, but some inflict systemic disease. The prevalent fish diseases in fish farms are usually initiated by bacteria. *Streptococcus spp* and *Proteus spp* among others are important fish pathogenic bacteria, causing bacterial infections and associated economic losses in fish culture worldwide (Akinyemi and Yekeen, 2010).

Fish diseases are responsible for heavy mortalities in fishes. Disease signs may include: erosion of the head (exposing the brain), fins and tail (exposing the backbone); eye damage (bulging eyes, blood spots or eroded eyes); gill erosion/fusion (the fish can't breathe properly); surface blood spots (i.e. haemorrhaging, ulceration, abscesses,

blisters/boils) and distended abdomen full of a straw-coloured sticky fluid (ascites). Internally, there may be haemorrhaging, a swollen digestive tract full of liquid, indicative of gastroenteritis, a swollen kidney, liver and/or spleen, liquefaction of the internal musculature and/or organs and the presence of tumours and/or granulomas. Vibriosis is a common disease of marine fish worldwide, with the signs appropriately described as that of a haemorrhagic septicaemia, i.e. there is abundant surface (particularly around the base of the fins) and internal (often in the musculature) haemorrhaging and necrosis/liquefaction of the organs (especially the kidneys). With advanced cases of disease, the pathogen is rampant throughout the infected animal according to Austin and Austin (2007).

According to Cabello (2006), the range of fish pathogens is steadily increasing and tropical examples include *Piscirickettsia salmonis* (salmonids rickettsial septicaemia; a disease which was first reported in Chile), *Moritella viscosa* (winter ulcer disease; this occurred originally in farmed salmon in Iceland) and *Pasteurella askyensis* (first isolated from Atlantic salmon in Scotland). Apart from the newcomers, aquatic animals have been subjected to many bacteria (*Aeromonas*, *Chryseobacterium*, *Flexibacter*, *Photobacterium*, *Pseudomonas*, *Renibacterium*, *Streptococcus*, *Staphylococcus* and *Vibrio*), viruses [e.g. infectious salmon anaemia (ISA), viral haemorrhagic septicaemia (VHS), infectious pancreatic necrosis (IPN), infectious haematopoietic necrosis (IHN)] and eukaryotic parasites (including sea lice and flukes)

Boyd *et al.*, (1977) described bacteria as single celled organisms which reproduce by binary fission and occur in various shapes

of rod (bacillus), spherical (coccus), comma (vibrios) and helical (spirillum). Most bacteria pathogenic to fishes are rod-shaped, only a few species of spherical pathogen are known to be pathogenic to fishes and there are no helical forms. Many bacteria pathogenic to fishes are psychrophilic. Only a few species will grow above 35°C. Most have a maximum growth temperature of 35°C or less. Many of the psychrophilic species have an optimum growth temperature of 10°C and continual replicate at 4°C.

Antibiotics are drugs of natural or synthetic origin that have the capacity to kill or to inhibit the growth of micro-organisms. Antibiotics that are sufficiently non-toxic to the host are used as chemotherapeutic agents in the treatment of infectious diseases of humans, animals and plants. They have long been present in the environment and have played a crucial role in the battle between man and microbe. However, Drug choices for the treatment of common infectious diseases are becoming increasingly limited and expensive and, in some cases, unavailable due to the emergence of drug resistance in bacteria and fungi – resistance that is threatening to reverse much medical progress of the past 50 years. Dissemination of resistant micro-organisms may occur in both hospitals and communities. It is recognized that a major route of transmission of resistant microorganisms from animals to humans is through the food chain (Akinyemi and Fajana, 2011). This study investigates the sensitivities of some synthetic antibiotics on *Streptococcus pneumoniae* and *Proteus mirabilis* responsible for mass mortalities of adult fish in fish tanks at Ikoto, Odogbolu Local Government Area.

Materials and methods

Study area

The study was carried out at Noble Fish Farms, Ijebu-Ode, Ogun State, Nigeria located on geographical grid on latitude $06^{\circ}12'$ North and longitude $3^{\circ}14'$ East (using Geographical Positioning System [GPS] Model – GARMIN GPSMAP 76CSX).

Samples collection

Carcasses of catfishes and water samples from fish tanks belonging to Noble Fish Farms, Ijebu-Ode were randomly collected for the study. They were transported to the laboratory in a sterile polythene bag immediately they were collected for bacteriological examination. Water samples were aseptically collected by the use of sterile sampling bottles from ponds where there are cases of fish diseases and examined for presence of microbes.

Gills and skin were collected aseptically using sterile scarpel after the fish carcass has been obtained. Each organ was aseptically collected into a sterile McCartney bottles, labeled A and B, being gills, and skin respectively.

Water samples were aseptically collected into sterile universal containers and labeled, the water samples were transported to the Laboratory for bacteriological examination, immediately they were collected.

Laboratory analyses of collected samples

Isolation of bacteria

Fish specimen: One gramme (1g) of each of the fish specimen was emulsified and thoroughly mixed in 10ml of sterile distilled water to have stock culture.

Water samples: One milliliter (1 ml) of each of the water sample was thoroughly mixed in 10ml of sterile distilled water to have stock culture.

Serial dilution

One milliliter (1 ml) of the stock culture was diluted with 9ml of sterile water aseptically

in a bottle which was then shaken together to make serial dilutions of 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} . One milliliter (1 ml) of the stock culture was diluted with 9ml of sterile water aseptically in first bottle with the aid of a sterile pipette to make 10^{-1} dilution (WHO, 1989). After this, another 1ml of the diluents was taken from the 10^{-1} dilution bottle to be transferred aseptically into another bottle containing 9ml of sterile water to obtain 10^{-2} dilution of sample. This was repeated to get the 10^{-6} dilution.

Pour plating and plate count

Onto already sterilized Petri dishes, 1ml of 10^{-6} dilutions of the sample were pipetted and the plates labeled. Thereafter, sterilized MacConkey Agar was poured onto the samples and swirled gently. The plates were allowed to gel and then incubated overnight at 37°C for microbial growth. Plate count was done manually by counting the number of colonies and the result multiplied by the dilution factor.

Purification of bacteria

Different bacterial colonies including *Streptococcus spp*, *Proteus spp*, *Pseudomonas spp*, *Klebsiella sp*, *E. coli*, *Staph aureus*, and *Bacillus subtilis* of the microbial growth were picked with sterile platinum wire loop and streaked on sterile MacConkey agar. These plates were incubated at 37°C for 24hours for microbial growth. Distinct colonies were re-inoculated on to fresh sterile Nutrient agar by streaking until pure isolates were obtained for characterization and identification.

Characterization of the isolates

Bacterial isolates were characterized by standard microbiological methods including colonial (size, shape, colour, consistency, edges, elevation and opacity), morphological (Gram staining and arrangement/shape) and biochemical characterization (oxidase, indole, urease,

citrate, catalase, sugar fermentation, methyl-red and Voges-proskauer). The results of the various microbiological tests were read and interpreted as described by Bergey (1984)

Identification of Streptococcus pneumoniae and Proteus mirabilis

Bergey's Manual of Bacteriology was used as a guide to identify bacterial isolates present in the samples examined based on various microbiological characterization carried out. However, *Streptococcus pneumoniae* and *Proteus mirabilis* were seen to be constant and predominant bacterial isolates among others and are thus singled out for sensitivity test.

Sensitivity assay of Streptococcus pneumoniae and Proteus mirabilis

Turbidity standard for inoculums preparation

Mc Farland standard provides Laboratory guidance for the standardization of number of bacteria for susceptibility testing or procedures requiring a standardization of the inoculums (Fatema *et. al.*, 2007). To standardize the inoculums density for a susceptibility test using a modified method (Ochei and Kolhatkar, 2008), a BaSO₄ turbidity standard equivalent to 0.5 McFarland standards or its optical equivalent should be used. The standard was distributed into the screw cap tubes of the same size and volume as these were used to dilute or grow the bacterial inoculums. The tubes were sealed tightly to prevent loss by evaporation and stored in the dark at room temperature.

Antibiotics activities of synthetic antibiotics on Streptococcus pneumoniae and Proteus mirabilis

The antimicrobial activity of 10 commercially available antibiotics on *S. pneumoniae* and *P. mirabilis* isolated from catfish tank culture system in Ogun State collected during disease outbreaks in fish

farm, were compared by growth inhibition by the modified disk-agar diffusion method on Nutrient Agar as recommended by Stukus (1997). The following commercial disks of antimicrobial substances used were: Amoxycillin (25 µg); Gentamicin (10 µg); Nitrofurantoin (300 µg); Augmentin (30µg), Tetracycline (30µg) Naladoxic acid (30 µg), Cotrimoxazole (10 µg), Erythromycine (30 µg) Cloxacillin (30µg) and Ofloxacin (5µg) for the synthetic antimicrobial activities

Determination of antimicrobial activity of commercial antibiotics

The commercially available antibiotic discs were placed aseptically with the use of a forceps gently at the centre of Nutrient agar plates that have already been seeded by flooding with the test organisms. The plates were then incubated at 37°C for 24 hours. This was done in triplicate. Interpretation of the isolates as sensitive or resistance was based on zones of inhibition using a meter rule in accordance to the standard. The mean values were taken in millimeters (mm)

Data presentation

Various observed data of the inhibition were presented in tables and percentages to determine the differences between mean inhibition diameters.

Results

Morphological and biochemical characterization of bacterial isolates

The morphological and biochemical characterization of bacterial isolates from the fish and water samples is shown in Table 1. The total viable counts of bacterial isolates present in the fish and the tank water examined are summarized in Table 2.

Sensitivity of Streptococcus pneumoniae and Proteus mirabilis to synthetic antibiotics

Inhibition zones of ten (10) commercially

Table 1: Morphological and biochemical characterization of bacterial isolates

Isolate number	1	2	3	4	5	6	7
Gram reaction	-	-	-	+		+	-
Shape	Rod	Rod	Rod	Cocci	Rod	Rod	Cocci
Biochemical characterization							
Catalase	+	+	+	+	+	+	-
Coagulase	-	-	-	+	-	-	-
Motility	+	-	+	-	+	-	-
Indole	-	-	+	-	-	-	-
Oxidase	+	-	-	-	-	-	-
Citrate	+	+	-	-	-	+	-
Urease	-	+	-	-	-	+	-
Hydrogen Sulphide Production	-	-	-	-	-	+	-
Methyl-red	+	-	+	-	+	+	+
Voges proskeur	-	+	-	+	-	-	-
Glucose assimilation	+	+	+	+	+	+	+
Lactose assimilation	-	+	+	+	-	-	-
Sucrose assimilation	-	A	-	-	-	A	A
Mannitol	-	A	-	A	-	A	-
Organism suspected	<i>Pseudomonas</i> sp	<i>Klebsiella</i> sp	<i>E. coli</i>	<i>Staph aureus</i>	<i>Bacillus subtilis</i>	<i>Proteus mirabilis</i>	<i>Streptococcus pneumonia</i>

produced synthetic antibiotic discs on *S. pneumoniae* and *P. mirabilis* is presented in Table 3. Ofloxacin had the highest inhibition zone of mean inhibition diameter of 26mm (26.5%), 20mm (25.6%) around *Streptococcus* sp and *Proteus* respectively.

This was followed by Gentamycin with mean inhibition diameter of 24mm (24.4%), 19mm (23.2%) and 15mm (30.6%) around *Streptococcus* and *Proteus* respectively. However, Naladoxic acid, Augmentin, Cloxacillin and Amoxycillin

Table 2: Total viable counts of bacterial isolates in fish and tank water

Sample	Total viable count (X10 ⁶ CFU / G)	Organism
Skin	8.6	<i>Pseudomonas</i> sp., <i>Proteus mirabilis</i> , <i>Streptococcus pneumonia</i> , <i>Bacillus subtilis</i>
Gill	6.5	<i>Staphylococcus aureus</i> , <i>Proteus mirabilis</i> , <i>Streptococcus pneumonia</i>
Tank A	9.6	<i>Klebsiella</i> sp., <i>Proteus mirabilis</i> , <i>Streptococcus pneumonia</i>
Tank B	7.3	<i>E.coli</i> , <i>Pseudomonas</i> sp. <i>E.coli</i> , <i>Streptococcus pneumonia</i> , <i>Proteus mirabilis</i>

Table 3: Inhibition zones of nine (9) commercially produced synthetic antibiotics discs on *S. pneumoniae* and *P. mirabilis*

Antibiotics	Mean inhibition zones(mm) <i>S. pneumoniae</i>		<i>P. mirabilis</i>	
		Perc. (%)		Perc. (%)
Ofloxacin	26(S)	26.5	20(S)	25.6
Gentamycin	24(S)	24.4	19(S)	24.4
Erythromycin	16(S)	16.3	17(S)	21.8
Cotrimoxazole	10(R)	10.2	13(R)	16.7
Tetracycline	12(R)	12.2	10(R)	12.8
Nitrofurantoin	10(R)	10.2	9(R)	11.5
Cloxacillin	0	0	0 (R)	0
Augmentin	00(R)	0	0 (R)	0
Amoxycillin	0(R)	0	0(R)	0
Naladixic acid	0(R)	0	0(R)	0
Total	98	100	78	100

Note

R-Resistant, S-Sensitive, Breakpoint = 14mm, Sensitive > 14mm, Resistant < 14mm (according to CLSI, 2007 standard)

exhibited no zone of inhibition (0mm) around test organisms.

Discussion

The result derived from the characterization shows that there are Gram negative bacilli and Gram positive cocci. All the isolates were positive to Catalase test except *Streptococcus pneumoniae* and also, all were negative to Hydrogen sulphide production except *Proteus mirabilis*. However, *Streptococcus pneumoniae* and *Proteus mirabilis* vary in reactions to other tests. The biochemical test as described by Ochei and Kohaltkar (2008) indicated that *Streptococcus pneumoniae* is cocci in shape and arrangement and reacted positive to Gram reaction and methyl red tests but negative to other tests, produced acid in sugar fermentation test using glucose, sucrose but not in lactose sugar and manitol. Whereas *P. mirabilis* was observed to be rod like and reacted positive to citrate, urease, H₂S production, methyl red and catalase tests, but negative to Gram reaction, coagulase, motility, indole and oxidase tests. The organism also produced acid in sugar fermentation test using

glucose and sucrose sugars and manitol but not in lactose sugar. These results were confirmed by Bergey, 1984.

Effect of synthetic antibiotics on the fish pathogenic bacteria under laboratory condition provided useful information for treatment of bacterial fish diseases. Also, the emergence of multi-drug resistance in human and animal pathogenic bacteria including fish pathogens as well as undesirable side effects of certain antibiotics has triggered immense interest in the search for more potent antimicrobial drugs. *Proteus mirabilis* and *Streptococcus pneumoniae* among others were observed to be constant and predominant in all the samples examined. Therefore, they were suspected to be responsible for the mortalities in the culture media. Thus, *Proteus mirabilis* and *Streptococcus pneumoniae* were singled out for sensitivity test. Other isolates including *Pseudomonas spp*, *Klebsiella spp*, *E. coli*, *S.aureus* and *Bacillus subtilis* were present as opportunistic microbes and are considered of little or no interest to this study. Antibiotic sensitivity and resistance tests of *Proteus mirabilis* and *Streptococcus pneumoniae* was performed under *in-vitro*

condition to determine inhibitory efficacy of various antibiotics tested. Ofloxacin had the highest inhibition zones around the test organisms with average inhibition diameter of 26mm (26.5%) and 20mm (25.6%) on *S. pneumoniae* and *P. mirabilis* respectively. This was followed by Gentamicin with average inhibition diameter of 24mm (24.4%), and 19mm (24.4%) on *S. pneumoniae* and *P. mirabilis* respectively. Erythromycin was next with zone of inhibition 16mm (16.3%) and 17mm (21.8%) on *S. pneumoniae* and *P. mirabilis* respectively. However, Cloxacillin, Augmentin, Amoxycillin and Naladoxic acid had no inhibition (0mm) around the two organisms tested. Although Cotrimoxazole, Tetracycline and Nitrofurantoin had inhibition zones around the test organisms, but they are considered to be resisted by these organisms because the antibiotics inhibition zones around them were less than 14mm (CLSI, 2007).

The sensitivity of some organism like *Pseudomonas aeruginosa*, and *Aeromonas sobria*, among others which reacted negative to Gram reaction, positive to Catalase and Citrate tests to drugs like Ofloxacin and Gentamycin and resistant to drugs like Cloxacillin, Amyxocillin and Naladoxic acid had been reported by Akinyemi, (2012). *Proteus mirabilis* and *Streptococcus pneumonia* were discovered to show similar sensitivity to drugs mentioned above in this work.

Conclusion and Recommendation

This study provided information on the bacteria (*S. pneumoniae* and *P. mirabilis*) sensitivity to

(10) Synthetic antibiotics (Tetracycline, Erythromycine, Cotrimoxazole, Gentamycin, Naladoxic acid Nitrofurantoin, Augmentin Ofloxacin and Cloxacillin). This study confirms the

potency of Ofloxacin, Gentamicin and Erythromycin because they showed highest inhibition zones among the synthetic antibiotics used. Thus, the three drugs can be effectively used in combating *Streptococcus pneumoniae* and *Proteus mirabilis* infections in fish.

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