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Original Research Article

Evaluation of multiplex and uniplex anitibiotics sensitivity discs on bacteria isolated from *Clarias gariepinus* in fish ponds at Umuahia, Nigeria

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ABSTRACT

The aim of this study was to evaluate the antibiotics types that are more sensitive to bacterial isolated from *Clarias gariepinus*. Two antibiotics types; namely multiplex and uniplex antibiotic sensitivity disc were used. Disc diffusion technique as described by Kirby-white was employed. Ten antibiotics were used for the study. A simple random sampling method was used. Six fishes were sampled from 6 different ponds, and a total of 36 fishes were screened. Swab samples were inoculated into nutrient agar and incubated at 37^0C for 24 hours. Grown colonies were sub-cultured into different media, incubated at 37^0C for 24 hours to obtain pure culture/isolate. Five different bacterial were isolated namely: *Staphylococcus spp*, *Streptococcus spp, Escherichia coli, Salmonella spp* and *Pseudomonas spp*. *Pseudomonas spp* was more frequently isolated, while *Streptococcus spp* was the least. Ciprofloxacin (CPX) from multiplex exhibited the highest zone of inhibition at 32mm while Levofloxacin (LEV) exhibited the highest inhibition zones from uniplex disc at 32mm for *Stahphylococcus spp.* Levofloxain in uniplex exhibited higher zones of inhibition than multiplex disc for *Escherichia coli* and same is applicable with CPX and LEV for *Salmonella spp*. The multiplex and uniplex antibiotic sensitivity disc had zone of inhibitions, however, the uniplex exhibited a higher inhibition zone than the multiplex across the five different bacterial isolated. This may be due to good preservation and storage of the antibiotic disc before use.

Keywords: Bacterial, *Clarias gariepinus,* inhibition zones, multiplex antibiotic disc, uniplex antibiotic disc, pond.

INTRODUCTION

Antibiotic sensitivity disc are paper laden with antibiotics according to standard concentration set by the clinical laboratory standard institute (CLSI, 2010). The antibiotic sensitivity disc comes as complex of 8, 10, 12, 14 and 16 antibiotics hence described as multiplex disc or as single of 50 disc cartridges describe as uniplex (Maggio, 2018)

Disc diffusion antibiotics sensitivity test is a culture-based microbiology assay used in the diagnosis and drug discovery laboratories (Bauer *et al.,* 1966). In diagnosis laboratories, the assay is used to determine the susceptibility of bacteria isolated from a patient's infection to clinical approved antibiotics (EUCAST, 2021). The disc diffusion method consist of placing paper disc saturated with antimicrobial agent on a lawn of bacteria seeded on the surface of the agar medium, incubating the plate overnight (Zapardiel *et al*., (1994)). Inhibition zone is a circular area around the spot of antibiotic in which the bacteria colonies do not grow (Bhargar *et al*., 2019). The zones of inhibition can be used to measure the susceptibility of the bacteria towards the antibiotic. The size of the zone of inhibition around the disc is measured after overnight incubation (Christenson & Relich, 2018). The incubation time by Kirby-Bauer method is a standardized technique for rapidly growing pathogen (NCCLS, 1993), and zones of inhibition are measured using a manual calliper or a ruler. The zones should be read against the dark background using reflective light or from the front using reflective light (Martinez *et al*., 1995).

Inhibition zones are transformed to the mean inhibitory concentration (MIC). Mean inhibitory concentration (MIC) is the lowest concentration (in µg/mL) of an antibiotic that inhibit the growth of a given strain of bacteria (Andrew, 2001). Fish are consumed and are considered as a high source of dietary protein (Boyd & Clay, 1998). Several

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bacterial affect *Clarias gariepinus*, and in a study in Thailand, isolated bacteria include *Aeronmonas spp, Pesudomona spp, Edwardsiella, Cetrobacter freundii* and *Shewanellabaltica* (Wakzak*et al.*, 2017). Novoslavskij *et al*. (2016) reported frequent isolation of *Yersinia spp, Salmonella spp, Vibro spp* and *Clostridium botalinum*. In a study conducted in China, Petty & Floyed (2015) reported isolation of *Aeromonas spp, Salmonella spp, Streptococci spp* and *Edwardsiella spp* as the frequently isolated bacteria organisms in fish. Antibiotic therapy is recommended in fishes that are ill.

Bacteria are microscopic single celled organisms that exist in their millions, in every environment, both inside and outside other organisms (Willey *et al.,* 2015). Some bacteria that affect fish have been responsible for morbidity and mortality leading to economic waste. Some aquatic bacteria equally are the cause of zoonoses (Lowry & Smith, 2007). Some bacteria like *Flavobacterium columnare* is the causative agent of Columnaris disease. This bacterial affects the cultured and wild freshwater fish causing skin lesion, fin erosion and gill necrosis (Declercq*et al.,* 2013). The objective of this study is to find out the antibiotic disc type that is more sensitive for effective prescription of drugs for farm use.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS AND MANAGEMENT

Fishes from six different artificial ponds (catfish) were used for the study. The ponds were supplied with clean water from bore hole and kept in quite environment. The water was changed once in a week. The fishes were divided into six (6) groups. The fishes were fed with fish feed bought from commercially sellers in Umuahia, Nigeria. A simple random sampling method was employed. Six fishes were sampled from each pond. A total of thirty six fishes were sampled. Samples were collected from the gill and surface of the fish with a sterile swab. The operculum was raised to access the gills. Swab stick was used to soak the area and samples collected were inoculated into nutrient agar media using streaking method and incubated at 37^{O}C for 24hours. The colonies that grew were picked and inoculated into blood agar, eosin methylene blue (EMB) media, deoxycholate agar and salmonella shigella agar.

ORGANISM ISOLATION

The method of Foster, (1996) was used for isolation of *Staphylococcus spp and Streptococcus spp*. Colonies of the sample that grew on the nutrient agar were inoculated into blood agar and incubated at 37^{O}C for 24hours.

GRAM REACTION

Gram staining was as described (Gram, 1938). Two colonies of the isolate were picked using wire loop and placed on a grease free slide, three drops of crystal violet were pipetted into the slides. This was washed off after 60seconds. Thereafter, acetone was pipetted into the slide which was then washed off after 60 seconds. Two to three drops of iodine was pipette into the slide and was washed off after 30 seconds. Safranin (three drops) was pipette into the slide, and washed off after 60 seconds. A drop of methylene blue was applied as stain and then viewed under the microscope at ×40 magnifications. The isolates were subjected to different biochemical tests for confirmation.

CATALASE TEST:

An agar slant containing the test organism was flooded with several drops of H_2O_2, and observed for reactions

ISOLATION OF *E. COLI*

Escherichia coli was isolated according to the procedure of Lejeune *et al.,* (2001). Colonies of the suspected sample that grew on the nutrient agar was inoculated into eosin methylene blue (EMB) agar and incubated at 37^{O}C for 24hours

ISOLATION OF *PSEUDOMONAS SPP*

The technique of Iglewski (1996) was employed in the isolation of *Pseudomonas spp.* Colonies of the suspected sample that grew on the nutrient agar were inoculated into blood agar and incubated at 37^{O}C for 24hours.

ISOLATION OF SALMONELLA

The procedure according to Ryan *et al.* (2017) was used for isolation of Salmonella. Colonies of the suspected sample that grew on the nutrient agar were inoculated into deoxycholate agar and incubated at 37^{O}C for 24hours.

DISC DIFFUSION TEST

The diffusion test was carried out according to Bauer *et al*. (1966). The multiplex disc was a product of Upturn Laboratories Ltd in Nigeria and was sourced from the market, while the uniplex disc was cartridges of 50 obtained from Oxoid, United Kingdom. The bacterial isolate was grown in pure culture. Using a sterile swab, a suspension of the pure culture was spread evenly over the face of a sterile agar plate. The antimicrobial agent is applied to the centre of the agar plate using multiplex antibiotic disc, while for the uniplex antibiotic disc, a dispenser was used to pick each of the single antibiotic and seeded onto the plate in such a way that they were wide apart from each other to avoid possibility of interwoven inhibition activity between one antibiotic and the other. The drugs used and their concentrations are; ciproflox (10 µg), norfloxacin (10 µg), erythromycin (30 µg), ampiclox (20µg), chloramphenicol (30 µg),amoxil (20µg), levofloxacin (20 µg), gentamicin (10 µg), rifampicin (20 µg) and streptomycin (30 µg).The plate is incubated at 37^{O}C for 24hours and thereafter a reading was taken. With the aid of meter rule or a pair of divider, the

zone of inhibition was measured and this was determined by placing the divider at the center of the media to the edge of the area of clearance. The zones of inhibition were compared with that of the national committee for clinical laboratory standards (NCCLS) guideline (NCCLS, 1995).

STATISTICAL ANALYSIS

The results of inhibition zones obtained between uniplex and multiplex were subjected to a two-way analysis of variance (ANOVA) test

RESULT

On blood agar, presence of light to golden yellow pigment with absence of haemolysis macroscopically, grape-like cocci arranged in clusters microscopically, and biochemically, catalase positive reaction suggests *Staphylococcus spp. Streptococci spp* on blood agar appear as greyish-whitish, smooth and translucent colonies with zones of β-haemolysis. The cocci were arranged in chains and negative reaction to catalase confirms the organism to be *Streptococci spp*. The presence of green metallic sheen on eosin methylene blue was confirmatory of *E. coli. Pseudomonas spp* on blood agar appear as blue-greenish colour, with mucoid colonies and having a fruity odour. The appearance of red colonies with black centers on deoxycholate agar (DCA) as well as the agar plate turning red is indicative of *Salmonella spp*. Figure 1 shows both multiplex and uniplex antibiotic sensitivity test for test organisms with their zones of inhibition post incubation. Inhibition zone shown varies from one test organism to the other. *Pseudomonas spp* was most isolated from group 2 in all the fishes sampled (Table I). *Pseudomonas spp* was more frequently isolated in all the six groups with isolation rate of 52.7%. This was followed by *Streptococcus spp* and *Salmonella spp* which had an isolation rate of 16.7% and 13.8% respectively. *Staphylococcus pp* and *Escherichia coli* were the least isolated. The zones of inhibition of ciprofloxacine, erythromycin and levofloxacine CPX, E and LEV was 32mm, 24mm and 24mm for multiplex disc for *Staphylococcus spp*, while the uniplex sensitive disc had a zone of inhibition of 24mm, 20mm and 32mm (Table II). Levofloxacin (LEV) had inhibition zones of 20mm and 28mm respectively for multiplex and uniplex antibiotic sensitive disc for *Streptococcus spp* (Table III). The zones of inhibition of multiplex sensitive disc for CPX, RD and LEV were 24mm, 24mm and 20mm for *Escherichia coli*, while uniplex antibiotic sensitive disc for CPX, LEV, RD and E were 32mm, 28mm, 28mm and 20mm (Table IV). LEV and S exhibited an inhibition zone of 12mm and 8mm for the multiplex disc, but 16mm and 12mm for the uniplex disc for *Pseudomonas spp*. The breakpoint in all the drugs used to determine whether sensitive or resistance varies depending on the organism in question (Figure III).

Table I: Frequency and percentage of isolation of bacteria from six different ponds

Bacteria species	1	2	3	4	5	6	% isolate
Staph spp	-	-	-	+	-	+	5.6%
Strep spp	-	++	+++	-	+	-	16.7%
E. coli	-	-	+	-	-	-	2.7%
Salmonella spp	+	++	-	+	+	-	13.8%
Pseudomonas spp	++	++++++	+++	+++	++++	++	52.7%

The sign (+) represents the number or numbers of bacteria isolated from each group, Staph= staphylococcus, Strep= streptococcus

Table II: Multiplex and uniplex antibiotic sensitively test with zones of inhibition (mm) to *Staphylococcus spp.*

NB=norfloxacin, CH=chloramphenicol, CPX= ciprofloxacin, E=erythromycin, LEV= levofloxacin, CN= gentamicin, APX =ampicillin, RD=rafampicin,

Disc types	NB	CH	CPX	E	LEV	CN	APX	RD	AMX	S
Multiplex	0mm	0mm	32mm	24mm	24mm	4mm	0mm	4mm	4mm	8mm
Uniplex	0mm	0mm	24mm	20mm	32mm	12mm	0mm	0mm	8mm	6mm

Table III: Multiplex and uniplex antibiotic sensitivity test with zones of inhibition (mm) to *Streptococcus spp*

NB=norfloxacin, CH=chloramphenicol, CPX= ciprofloxacin, E=erythromycin, LEV= levofloxacin, CN= gentamicin, APX =ampicillin, RD=rafampicin, AMX=amoxil, S=streptomycin. P≤0.05

Disc types	NB	CH	CPX	E	LEV	CN	APX	RD	AMX	S
Multiplex	0mm	0mm	16mm	0mm	20mm	0mm	0mm	0mm	8mm	0mm
Uniplex	0mm	8mm	0mm	28mm	0mm	0mm	8mm	6mm	0mm	0mm

DISCUSSION

Fish is a good source of protein to humans and the need to eat wholesome fish is of immense importance for public health. Micro-oganisms are the major causes of foodborne

diseases, which have resulted in various forms of illness and death. From this study, five different bacteria were isolated from the study and these includes; *Salmonella spp, Staphylococcus spp, Streptococcus spp, Pseudomonas spp*

Table IV: Multiplex and uniplex antibiotic sensitivity test with zones of inhibition (mm) to *Streptococcus spp*
NB=norfloxacin, CH=chloramphenicol, CPX= ciprofloxacin, E=erythromycin, LEV= levofloxacin, CN= gentamicin, APX =ampicillin, RD=rafampicin, AMX=amoxil, S=streptomycin. P≤0.05

Disc types	NB	CH	CPX	E	LEV	CN	APX	RD	AMX	S
Multiplex	0mm	0mm	24mm	16mm	20mm	0mm	0mm	24mm	0mm	0mm
Uniplex	0mm	8mm	32mm	20mm	0mm	0mm	0mm	28mm	0mm	0mm

Figure 1: Multiplex and uniplex antibiotic sensitivity test showing zones of inhibition of different test organisms.

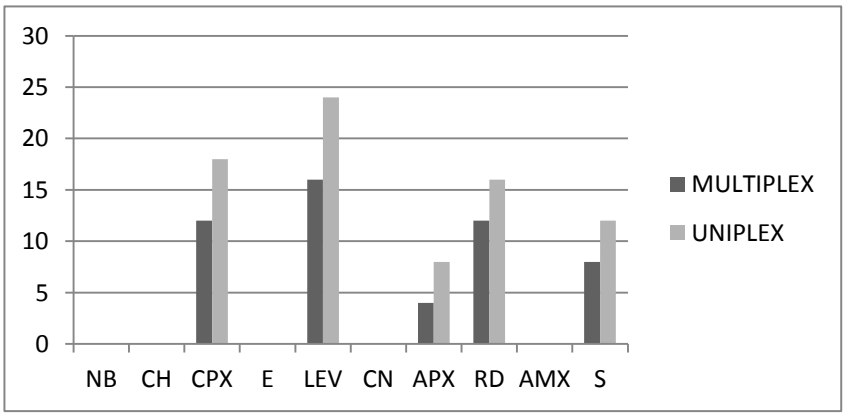

Figure 2: Multiplex and uniplex antibiotic sensitivity test with zones of inhibition (mm) to *Salmonella spp*. NB=norfloxacin,CH=chloramphenicol, CPX=ciproflox, E=erythromycin, LEV=levofloxacin, CN=gentamicin, APX=ampiclox RD=rifampicin, AMX=amoxil, S=streptomycin, inh.z-Inhibition zone $P \leq 0.05$

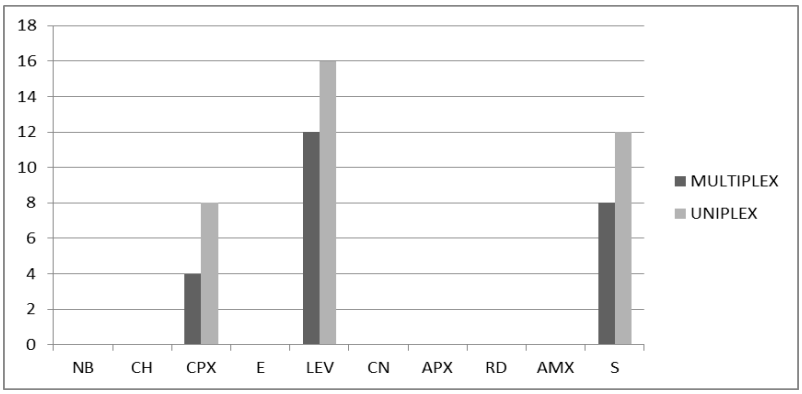

Fig III: Multiplex and uniplex antibiotic sensitivity test with zones of inhibition (mm) to *Pseudomonas spp.* NB=norfloxacin,CH=chloramphenicol, CPX=ciproflox, E=erythromycin, LEV=levofloxacin, CN=gentamicin, APX=ampiclox, RD=rifampicin , AMX= amoxil S=streptomycin, Inh.z=Inhibition zone, $P \leq 0.05$

and *Escherichia coli* and this agrees with earlier findings (Hodobo *et al.,* 2019). Hodobo *et al*., (2019), in his study in Mufakose market in Zimbabwe reported that *Escherichia coli, Staphylococus spp, Streptococcus spp, Pseudomonas spp* and *Salmonella spp* were frequently isolated. However, this finding is inconsistent with Joh *et al*. (2013) who reported that *Aeromona sspp* was the most frequently isolated. *Pseudomonas spp* was most frequently isolated (52%), *Streptococcus spp* (16.7%) while *Escherichia coli* was the least (2.7%) and this is in agreement with Wamala *et al.* (2018), who reported frequent isolation of same organisms (40%, (12.4% and 3%) respectively. However, it disagrees with the report of Hodobo *et al.* (2019) who opined that (3%), (6.3%) and (44%) of *Pseudomonas spp, Streptococcus spp* and *Escherichia coli* was isolated. The zone of inhibition is used to measure the susceptibility of the bacteria towards the antibiotics. The inhibition zones of multiplex and uniplex of a particular antibiotic used presented values with mild variation. The high inhibition zone of CPX, E and LEV at 32mm, 24mm and 24mm for multiplex antibiotic disc and 24mm,20mm and 32mm for uniplex antibiotic disc shows that both antibiotic discs exhibited similar reaction and are sensitive for *Staphylococcus spp* (Table II) with the breakpoint of 20mm for sensitive and less for resistance as supported (CLSI,2012). Only LEV had a high inhibition zone of 20mm and 28mm for multiplex and uniplex antibiotic sensitivity test (Table III) for *Streptococcus spp* and this agrees with the findings of Gonzalez-Rey *et al*. (2004), but disagrees with Rothenburger *et al*. (2002). High zones of inhibition was exhibited by CPX, RD and LEV at 24mm, 24mm and 20mm for multiplex

disc while, 32mm, 28mm and 28mm for uniplex disc. The zones of inhibition exhibited by these antibiotics shown in figure 2 and 3 was against the null hypothesis $P \leq 0.05$, when subjected to a two way ANOVA. There was no significance difference between the multiplex and uniplex antibiotic disc in their reactions to various bacterial organisms. The five bacterial did not exhibit sensitivity to the following antibiotics; nor-floxacine, chloramphenicol, erythromycin, ampiclox, rifampicin and amoxil as there were no visible zones of inhibition. The resistance of *Escherichia coli* and *Salmonella spp* to ampicillin, erythromycin, nor-floxacin and amoxil is consistent with the findings of Adzitey (2018). In this study, LEV and CPX inhibition zones were 24mm and 20mm for the uniplex and this is in agreement with Chand *et al.* (2014), who in their study in Napel, reported that ciprofloxacin had a high inhibition zone to these bacterial, but in disagreement Sjolund-karlisson *et al.* (2014), who stated that ciproflox and levoflaxacin had inhibition zones of 30mm and 28mm respectively, with $\geq$ 28mm and $\geq$ 25mm as the breakpoint that determines sensitivity for salmonella. CPX resistance to *Streptococcus spp, Salmonella spp* and *Pseudomonas spp* for both multiplex and uniplex antibiotic disc with inhibition zones of 16mm, 12mm and 4mm was consistent with Ali *et al*. (2010) who reported low inhibition zones ranging from 8mm to 12mm for these organisms. All the ten drugs use for this study for multiplex and uniplex had inhibition zones that ranged from 4mm to 16mm and this was supported by Hiby & Sams (2019), who reported an inhibition zones of between 10mm and 14mm for *Pseudomonas spp*.

CONCLUSION

Both multiplex and uniplex antibiotic sensitivity discs were effective in determining the susceptibility test of bacterial isolates. The inhibition zones exhibited by uniplex antibiotic disc were more pronounced than the multiplex disc. The uniplex sensitivity disc was slightly more sensitive than the multiplex disc and this may be due to method of storage by the distributing companies.

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CONFLICT OF INTEREST

There is no conflict of interest to declare.

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