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MULTIPLE ANTIBIOTIC RESISTANCE INDICES OF *AEROMONAS HYDROPHILA* ISOLATES OF MUSCLE OF CATFISH (*CLARIAS GARIEPINUS*, BURCHELL 1822) FROM SELECTED MARKETS IN IBADAN, NIGERIA

[*]Saka, B. A., [*]Adeyemo, O. K.&[+]Odeseye A. O

[*]Fish and Wildlife Unit, Department of Veterinary Public Health and Preventive Medicine University of Ibadan, Ibadan, Nigeria; [+]Nigerian Institute of Science Laboratory Technology, Ibadan

Correspondence sakababatunde@gmail.com

ABSTRACT

The extensive use and misuse of antimicrobials for treatment and prophylaxis in livestock production generally and aquaculture in particular is of great concern to environmental and public health. In Nigeria, regulation and monitoring of aquaculture and other livestock production activities at best is lax. Drug resistance pathogens have therefore been consistently reported in Nigeria.

Ninety-eight adult live fishes weighing an average of 684.88±141.73g were purchased at random from different live-fish selling points fortnightly over a fourteen-week period. Fish were anaesthetized using Tricaine Methane Sulfonate (MS222 and 15g of muscle excised and processed according to standard methods. Growth, isolation and characterization of *Aeromonas hydrophila* was accomplished using Rimler-Shotts agar medium which had been infused with ampicillin supplement for 24 hours and incubated at 37oC and appropriate biochemical tests.

Ten positive isolates (AH1-AH10) were subjected to culture and sensitivity test using the disc diffusion method on nutrient agar. Zones of growth inhibition around the colonies were observed, measured and characterized as sensitive, intermediate and resistant based on the Manual of Antimicrobial Susceptibility Testing method. All the isolates had MAR >0.2. Isolate AH9 had the highest MAR index (1). Three of the isolates (AH3, AH5 and AH8) had MAR indices of 0.89, while AH2, AH4 and AH7 had MAR indices of 0.67. This study established the resistance of *Aeromonas hydrophila* isolates from fish muscle to a wide range of antibiotic. The detection of high MAR *A. hydrophila* in muscle of fish intended for consumption is significant and could act as a potential source of resistant bacteria for humans. Further investigation into antimicrobial resistance is recommended.

PLUSIEURS INDICES DE RÉSISTANCE AUX ANTIBIOTIQUES DE *AEROMONASHYDROPHILA* ISOLE OFMUSCLE POISSON-CHAT (*CLARIASGARIEPINUS*, BURCHELL 1822) DE CERTAINS MARCHÉS À IBADAN (NIGÉRIA)

*** Saka, B. A., * Ali, O. K. &[+] Odeseye A. O**

*** Des poissons et la faune Unit, Department of Veterinary Public Health et médecine preventive, Université d'Ibadan, Ibadan, Nigeria; [+] Nigerian Institute of Science technologie de laboratoire, Ibadan**

Correspondance sakababatunde@gmail.com

RÉSUMÉ

L'utilisation intensive, la mauvaise utilisation des antimicrobiens pour le traitement et la prophylaxie en élevage en général et de l'aquaculture en particulier est de grande préoccupation pour l'environnement et la santé publique. Au Nigeria, réglementation et surveillance de l'aquaculture et des autres activités de production de bétail au mieux est laxiste. Pathogènes de résistance de drogue ont donc été toujours déclarés au Nigeria.

Quatre-vingt-dix-huit des poissons vivants adultes pesant en moyenne 684.88±141.73 g ont été achetées au hasard à différents points de vente de poissons vivent toutes les deux semaines sur une période de quatorze semaines. Poissons ont été anesthésiés à l'aide de méthane-Sulfonate de tricaïne (MS222 et 15g de muscle excisé et préparée selon les méthodes standard. Croissance, l'isolement et caractérisation des *Aeromonashydrophila* a été réalisée à l'aide de milieu gélosé Rimler-Shotts qui avait été imprégné de supplément ampicilline pendant 24 heures et incubé à 37 ° c et des tests biochimiques appropriés.

Dix isolats positifs (AH1-AH10) ont été soumis à essai de culture et antibiogramme à l'aide de la méthode de diffusion des disques sur la gélose nutritive. Zones d'inhibition de croissance autour des colonies ont été observées, mesurés et caractérisés comme sensible, intermédiaire et résistant basé sur la méthode du manuel des tests de sensibilité aux antimicrobiens. Tous les isolats avaient MAR > 0,2. AH9 isolat avait le plus fort indice de MAR (1). Trois des isolats (AH3, AH5 et AH8) avaient des indices de MAR de 0,89, tandis que AH2, AH4 et AH7 avaient MAR indices de 0,67. Cette étude a établi que la résistance de *Aeromonashydrophila* isole du muscle de poisson à une vaste gamme d'antibiotique. La détection de haute MAR *A.hydrophila* dans le muscle de poisson destinés à la consommation est importante et pourrait agir comme

une source potentielle de bactéries résistantes pour les humains. Enquête plus approfondie sur des gènes résistants aux antimicrobiens provenant de cette source est recommandée par les présentes.

INTRODUCTION

Aeromonas hydrophila is an ubiquitous organism ranked as the most common bacteria in freshwater habitats all over the world. It is also represented as a part of the microflora of the intestine of healthy fish. However, these bacteria frequently cause disease among cultured and feral fishes as soon as there is an imbalance in a pond set-up or an introduction of any of the stress factors. Members of this group exhibit diverse genetic, biochemical and antigenic heterogeneity and therefore forms a complex of disease organisms that are associated with bacterial hemorrhagic septicemias and other ulcerative conditions in fishes and humans-making it zoonotic.

Motile aeromonad septicaemia has been demonstrated in both fresh- and brackish-water fish species, marine fishing grounds and raw and processed products of marine fish [1, 2, 3, 4, 5].

Aeromonas hydrophila occurs as a Gram-negative, motile, straight rod (0.3-1.0x 1.0-3.5µm). It forms white to buff, circular, convex colonies within 24 hours at 22- 28oC. Rimmler-shotts infused with ampicillin or novobiocin has been found useful for the isolation and presumptive diagnosis of *A. hydrophila*. It is a cytochrome oxidase-positive organism resistant to the vibriostatic agent 0/129. It ferments glucose with or without the production of gas and this differentiates it from Pseudomonas [6, 7].

Its possession of two classes of adhesins permits it to bind to specific receptors on cell surfaces and these are described as adhesins associated with filaments and those associated with proteins. The filaments are also involved in phage-binding biofilm formation as well as twitching motility and are distributed in different proportion on the different strains of aeromonads. The variation in the distribution of the pili is suspected to be [18] causing enteric infections such as traveller's diarrhoea, severe diarrheal disease of short duration or chronic loose stools in children, the elderly, or immuno-compromised individuals [19]. Dermal infections such as cellulitis contracted from fish handling [20], septicemia sustained due to immunodeficiency from other protracted illness, urinary tract infections, surgical wound infections, meningitis, peritonitis, and endocarditis have been associated with *Aeromonas hydrophila* infection in humans [21]. It is important to note that Aeromonas species have also been reported to have the inherent capability to be resistant to chlorination [22]. *Aeromonas hydrophila*is listed in the Contaminant Candidate List, and Environmental Protection Agency Method 1605 has further validated its detection and enumeration in drinking water system [23].

responsible for the behaviours of the bacteria such as seen when motile aeromonads taken from lesions on diseased fish shows a greater chemotactic response to skin mucus than isolates that were obtained as free-living organisms from pond water [8]. [9] also indicated that *A. hydrophila* had adhesive agglutination characteristics which facilitated attachment to eukaryotic cells. [10] showed that the presence of a 52kD surface protein in in the S-layer has been responsible for an increase in their cellular hydrophobicity which enhances resistance of the bacterium to serum lysis and phagocytosis by leukocytes. [11] and [12] have documented that many of the aeromonads possessed hemorrhagic factors and lethal toxins at different concentrations. Further interest in the virulence of *A. hydrophila* was elicited when it was revealed that enterotoxins, haemolysins, proteases, haemagglutinins, and endotoxins produced by bacterial organisms exhibit synergism in inducing clinical pathology [13, 14, 15].

In the acute or severe form of the disease, morbidity and mortality is high and sudden, clinical signs include exophthalmia, reddening or darkening of the skin, and fluid accumulation in the scale pockets [16]. There may be ascites and the scales may bristle out from the skin to give a "washboard" appearance. The gills may show varying degree of haemorrhage and ulcers may develop on the dermis. Dermal ulcers form shallow necrotic lesions, internal organs are swollen and congested with haemorrhages over the viscera. The kidney and swollen spleen usually contain a semi-fluid which may drip out.

In humans, Aeromonas species have been listed as a pathogen for various diseases in man [17]. It has been shown as food and water borne pathogen of significance

Aeromonas hydrophila and antimicrobial use

Antimicrobials have been in use to control the incidence of bacterial diseases in aquaculture for a long time. In the 1980s, Norway experienced huge losses in its fish industry due to bacterial diseases and a total crash in the industry was only prevented by the use of vast quantity of antibiotics [24].

The use of antimicrobial agents in aquaculture and its potentials to contribute to antimicrobial resistance raises important issues relating to public health, food safety, animal health and production. Due to non-existence of rules curbing the indiscriminate use of antimicrobials in aquaculture [25, 26, 27] and other livestock production [28, 29, 30] in developing countries or the lack of enforcement in places where such rules exist; antimicrobial are used without caution or consideration of the adverse effect on public health with regards to development of drug resistance. This study was designed to determine Multiple Antibiotic Resistance (MAR) indices of *Aeromonas hydrophila* isolates in the muscle of African sharptooth catfish (*Clarias gariepinus*, Burchell 1822) from selected live-fish markets in Ibadan, Nigeria.

MATERIAL AND METHODS

To ensure fishes were not from the same source in Ibadan metropolis, ninety-eight adult live fishes weighing 684.88±141.73g at the average were purchased at random from seven live-fish selling points fortnightly over a fourteen-week period. Fish were anaesthetized using Tricaine Methane Sulfonate (MS222), then15g of muscle was excised from the mid-region and homogenized with 30ml of alkaline peptone solution (Oxoid, UK). Homogenates were left at room temperature for 24 hours after which aliquots were taken and inoculated in Rimler-Shotts agar medium which had been infused with ampicillin supplement for 24 hours and incubated at 37oC. Bacterial growth was harvested and subjected to biochemical tests to identify and isolate *Aeromonas hydrophila* from the samples.

Culture and sensitivity test was carried out using the disc diffusion method on nutrient agar. Zones of growth inhibition around the colonies were observed and measured and then characterized based on the Manual of Antimicrobial Susceptibility Testing method as sensitive, intermediate and resistant. The sensitivity disc used was from Abtek Biologicals UK, LOT QE06/PB/P. The choice of antibiotic was based on the frequently used antibiotics for treatments of infection in local clinics [31] as well as antibiotics of choice used by farmers during fish rearing [27].

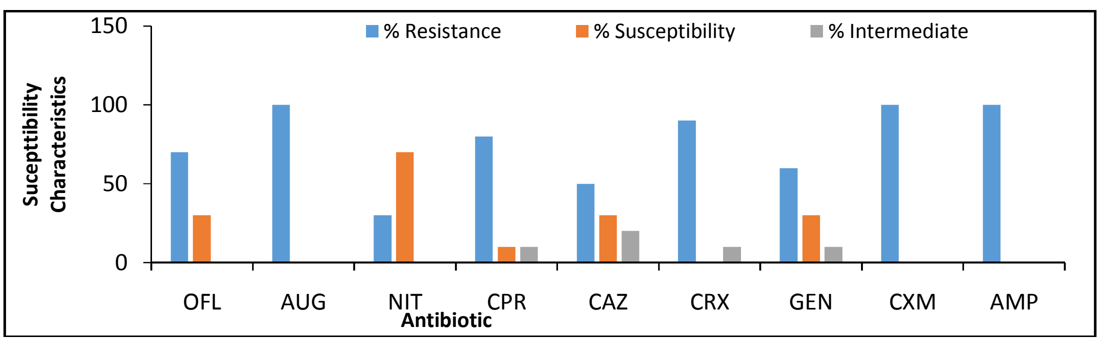
Multiple Antibiotic Resistance Indexing
The MAR index in individual isolates was calculated as a/b according to [32], where:

- a represents the number of antibiotics to which the isolate was resistant to
- b represents the number of antibiotics to which the isolate was exposed.

An MAR index higher than 0.2 was considered to be high-risk, with possible exposure to high doses of antibiotics over a period of time. While a MAR index less than or equal to 0.2 was considered as an original strain resistance to the antibiotic without previous exposure to such antibiotic.

RESULTS

A total of ten *Aeromonas hydrophila* isolates (AH1-AH10) were obtained and tested for antibiotic susceptibility during the sampling period. All the isolates showed varying degree of multiple resistant pattern to more than one antibiotic. All the isolates showed 100% resistance to Ampicillin, Cefixime and Augmentin, resistance to ciprofloxacin was 80% while it was 90% for cefuroxime. Nitrofurantoin had the highest susceptibility at 70%, while (30%) were susceptible to Ofloxacin, Ceftazidime and Gentamycin.



OFL- Ofloxacin, AUG- Augmentin, NIT- Nitrofurantoin, CPR- Ciprofloxacin, CAZ- Ceftazidime, CRX- Cefuroxime, GEN- Gentamycin, CXM- Cefixime, AMP- Ampicillin

FIG. 1: SHOWING THE SUSCEPTIBILITY CHARACTERISTICS OF SOME *AEROMONASHYDROPHILA* TO SELECTED ANTIBIOTICS

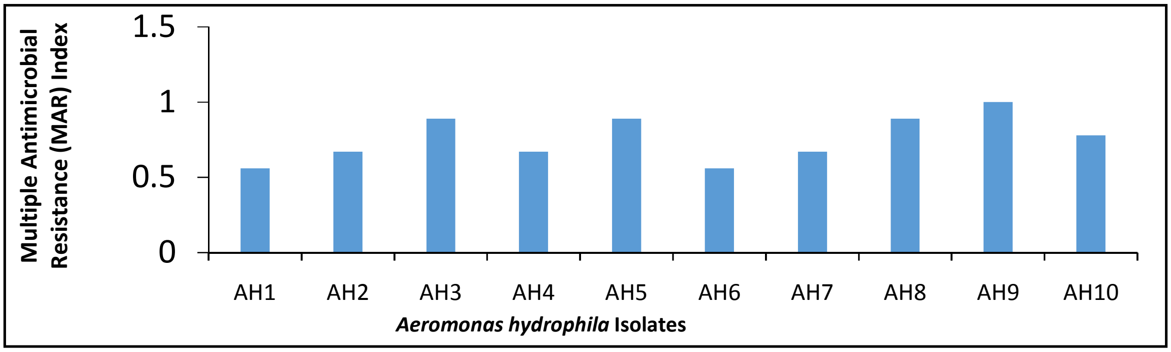


FIGURE 2: MULTIPLE ANTIMICROBIAL RESISTANCE (MAR) INDICES OF *AEROMONASHYDROPHILA* ISOLATES FROM *CLARIASGARIEPINUS'* MUSCLES

DISCUSSION

In this study, all the isolates (100%) had multiple antibiotic resistance (MAR) indices >0.2 (Figure 2). Isolate AH9 had the highest MAR index (Figure 1). Three of the isolates (AH3, AH5 and AH8) had MAR indices of 0.89, while AH2, AH4 and AH7 had MAR indices of 0.67. This agrees with some studies where the capabilities of these bacteria to develop resistance and transmit such effectively have been established [33, 34]. Ofloxacin is a fluoroquinolone which is supposed to be active against skin infection such as cellulitis caused by *Aeromonas hydrophila* in humans but no report of its potency against *Aeromonas hydrophila* has been documented. It is further categorized as 'of high regulatory concern' by the FDA in the United States and its use to treat any food animal is regarded as illegal and completely irresponsible [35], yet the level of resistance is highly significant in *Aeromonas hydrophila* from this study. The resistance to augmentin (100%) recorded in this study agrees with records of [36] who also recorded 100% resistance to Ticarcillin/clavulanic acid and 92.6% to Amoxillin/Subactam (AMX-SUL) by Trifamox IBL(Laboratories Bagó, Buenos Aires, Argentina). Nitrofurantoin demonstrated the highest susceptibility (70%) in all isolates tested, this is close to a previously report of 100% by [34] but contrary to that of [37] who reported 42% susceptibility to nitrofurantoin. Such differences may be clearly demonstrates the kinetic ability of bacterial organisms in developing resistance to antimicrobials. The nitrofurans, which include nitrofurantoin, nitrofurazone, furanace, and furazolidone, are frequently used to treat pet and ornamental fishes in developed nations; it is strictly forbidden for use in food fish by the FDA in the US [35]. However, the same cannot be said of Nigeria where there is indiscriminate use of antibiotics in food animals and fishes because of the inadequate regulations and lack of enforcement of existing regulations. Antimicrobials are dispensed without proper diagnosis and prescription by veterinary personnel [38, 39, 40] reported that nitrofurans are not readily absorbed into the body in gilthead sea bream (*Sparusaurata*) and tilapia (*Oreochromis mossambicus*); therefore, their efficacy is seen more in superficial infections. Nitrofuran is also known to be reactive to light therefore there is the need to administer it under the cover of some form of darkness to achieve the most effective result. Therefore even the recorded susceptibility is not so good news for food fishes.

Records show that aminoglycosides such as kanamycin and gentamycin have broad spectrum activity against Gram negative and gram positive bacterial organisms. However, it is to be noted that the reported 100% efficacy of gentamycin were from those administered via injection and it has also been reported that such administration predisposes to kidney damage in fishes [35]. Gentamycin resistance (60%) and susceptibility (30%) in this work therefore calls for concern because aeromonads has been largely reported to be sensitive to gentamycin although there are reports of resistance as well [41].

Presence of antibiotics in aquatic bodies starts with natural production. The presence of the group of Actinomycetes which includes Streptomycetes produces antibiotics, several antibiotics such as some ß-lactams, streptomycins, aminoglycosides and others are produced by soil bacteria [42]. This antibiotic activity from local soil samples is variable and as such naturally occurring bacterial presence is seen more in tropical soil and by extension aquatic bodies as tropical soil are known to possess more bacteria producing antibiotics especially for tetracycline [42].

However, antibiotics usage has been largely responsive for the development of resistance in the bacterial pathogens. Extensive usage in human and indiscriminate usage in veterinary practise especially for the purpose of preventing (prophylaxis) or treating microbial infections. International usage of antibiotics is also not harmonised therefore antibiotics prohibited in some countries are permitted in others such as the use of streptomycin in fruit growing in the USA meanwhile this is prohibited in Germany. Utilization as growth promoters also has its contribution to bacterial resistance. Of the 10,200 tons of antibiotics utilized in the EU in 1996, 50% was applied in veterinary medicine and as growth promoters. Data such as that of the European Federation of Animal Health [43], revealed that a total of 13,216 ton of antibiotics were used in the European Union and Switzerland, 65% of which was applied in human medicine in 1999 [42]. Interestingly, [44] and [45] revealed that contrary to expectation, hospitals are not the main source of pharmaceuticals in municipal sewage. Rather, community use is largely responsible supported by records such as 70% in the UK [46], 75% in the US [47] and 75% in Germany [48].

Metabolism of these antibiotics in human and animals vary a lot [49, 48]. Some compounds are metabolized by 90% or more, while others are metabolized by only 10% or even less. The excretion rates for the unchanged active compound for most of the antibiotics also remains in the same range of between 10%–90%. [48] recorded an average of 30% metabolic rate for all antibiotics used leaving 70% of the used antibiotics excreted unchanged into waste water with every likelihood that the metabolites are more water soluble than the parent compounds. It is also common practise to flush unused drugs including antibiotics down into the toilet.

In the developed nations, attempts are made to remove these antibiotics from the environment during sewage treatment but this only reduces the volume on such antibiotics and partially eliminates them. Therefore these antibiotics end up in the environment and precisely in aquatic bodies.

Conclusion

This study has established that all the strains tested showed a MAR index value greater than 0.2, thus indicating that the isolates are from high risk sources, such as sewage, animal husbandry waste, faecal contaminated drinking water. Organic feeding of chicken waste, chicken entrails as well as blood meal has been implicated in the transmission of such antimicrobial exposed aeromonads to fish. The detection of *A. hydrophila* in muscle of fish intended for consumption is significant and grave as it could act as a potential source of resistant bacteria for humans. Similar studies all over the world have consistently documented multiple **antibiotic resistance** to *A. hydrophila* [50, 36, 37, 51,34].This study also further corroborates the reports of excessive and uncontrolled use of antimicrobials in Nigerian aquaculture [27] and other livestock industries [28, 29, 30] leading to the development of antimicrobial resistant pathogens.

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