The Antibiotic Resistant Patterns Of Bacterial Flora Of Cultured Catfish Fed With Poultry Hatchery Waste From Selected Farms In Ibadan, Nigeria

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Abstract: Microbial quality of poultry hatchery wastes from three selected commercial poultry hatchery units and catfish (Clarias gariepinus) fed hatchery waste obtained from five purposively selected aquaculture farms in three local government areas in Ibadan, Oyo state, Nigeria were studied using standard microbiological methods. The antibiotic sensitivity test of the isolated organisms was also carried out to determine their sensitivity to seven different antibiotics commonly used by livestock farmers. The antibiotics were Nitrofuratoin (F), Augmentin (AMC), Ciprofloxacin (CIP), Nalidixic acid (NA), Erythromycine (E), Chloramphenicol (C) and Gentamicine (CN). The results of the samples examined were compared using both descriptive and inferential statistics. One-way analysis of variance was used to compare means among the three different hatcheries and organs. The level of significance was set as p < 0.05. The total bacterial count obtained ranged from 1.2 x 10^5 to 4.6 x 10^5 cfu/g and 1.2 x10^5 to 5.6 x 10^5 cfu/g for the hatchery waste and catfish respectively. The Total enterobacterial count ranged from 6.0x10^4 to 3.0x 10^5 cfu/g and 4.0x104 to 3.0x10^5 cfu/g for hatchery waste and catfish respectively. The Bacteria isolated from hatchery waste were Staphylococcus epidermidis, Esherichia coli, Bacillus spp, Klebsiella pneumonia and Pseudomonas aeruginosa, while those isolated from different organs (skin, stomach and intestines) of the catfish were Salmonella subsp1, Leclercia adecarboxylata, Bacillus spp, Klebsiella pneumonia, Eschericia coli, and Staphylococcus aureus, Citrobacter spp, Pseudomonas aeruginosa. Salmonella arizonea subsp3A. There were frequent occurrence of Escherichia, Staphylococcus, Pseudomonas, Salmonella, & Klebsiella in the samples (hatchery waste and catfish organs) analysed. E coli were sensitive to all the antibiotics except Erythromycin. Salmonella arizonea subsp.3 was also sensitive to all the antibiotics except Nalixidic acid, Erythromycin, and Augmentin. Erythromycin was not active against any of the isolated organisms while Bacillus spp was not sensitive to any of the antibiotics. Gentamycin showed highest level activity against all the isolates while Erythromycin showed no activity against any of the isolates. This level of resistance to antimicrobial agents is a reflection of misuse or abuse of these agents in the environment.

INTRODUCTION
Population growth is accompanied by increasing demand for food. Direct human consumption of fish reached an estimated 103 million tons in 2003 (World Fish Center, 2009). Fish and fish products constitute more than 60% of the total protein intake in adults especially in the rural areas where they are widely accepted and form a much-cherished delicacy that cut across-economic, age, religious and educational barriers (Adeleye, 2003). Fish has always been a source of “rich food for poor people” and played an important role in improving a developing country’s food security and nutrition for its people. Fish is a major source of high-quality dietary protein, essential vitamins, minerals, and other micronutrients for about 1 billion people, many isolated in rural communities of developing and low income countries (Toft, 2001 and FAO, 2003). About 25% of animal protein is obtained from fish and shell fish and this is required by the body for growth and maintenance of lean muscle and tissue (Ayyoola, 2011).

Lack of nutrients is major constraint to aquaculture development and this remains true for much of the catfish culture practised in the developing world. Livestock production systems, and opportunities for reuse of wastes and byproducts, are changing. The intensive nature of modern poultry production and processing tends to concentrate high quality byproducts, and this has stimulated their reuse as livestock feeds.

Concept of utilizing poultry wastes is highly desirable since it will not only eliminate problem of waste disposal but also provide cheap fish feed (Ayyoola, 2011). Poultry hatchery waste is an unconventional feed now increasingly used in freshwater aquaculture for economic reasons (Ayyoola, 2010). Poultry industry produces large
According to Apata (2009), it is the animal by-products, fungi, and viruses (from faecal matter) that may adversely affect the consumers’ health. The production system also presents risks to public health and major health risks of aquaculture products are biological, especially for the organisms produced in waste water or water receiving animal and human wastes. Safety of consuming fish products from such environments becomes questionable (Erondu and Ananwu, 2005). The overall sanitary quality of the waters from which these catfish are cultured is key to the overall microbial quality of their finished products. There is a serious risk of potential health hazards arising from pathogenic microbial contamination or parasites within the waste material and from these animal based feedstuffs. There is a possibility of a hazard arising from the presence of viable and contaminating micro-organisms, including bacteria (i.e. Salmonella contamination within animal by-products), fungi, and viruses.

In different studies, several types of microbial and bacterial pathogens have been associated with farmed fish. Many pathogenic microorganisms and parasites could conceivably be transmitted to humans through fish. (George, 2008). Bacterial flora isolated from eggs, skin, gills, and intestines have been described for a limited number of fish species. Generally, there is a relationship between the aquatic environment of the fish and the range of bacterial genera isolated and this varies with factors such as the salinity of the habitat and the bacterial load in the water. Bacteria recovered from the skin and gills may be transient rather than resident on the fish surfaces. The complexity of the fish digestive system appears to determine the microflora of fish intestines. The genera present in the gut seem to be those from the environment or diet which can survive and multiply in the intestinal tract, although there is evidence for a distinct intestinal microflora in some species. While obligate anaerobes have been recovered from carp and tilapia intestines, low ambient temperatures may prevent colonization by anaerobes in species such as rainbow trout (Cahill, 1990).

There is a serious risk of potential health hazards arising from pathogenic microbial contamination or parasites within the waste material and from these animal based feedstuffs. There is a possibility of a hazard arising from the presence of viable and contaminating micro-organisms, including; bacteria (Salmonella contamination within animal by-products), fungi, and viruses (from faecal waste products). According to Apata (2009) it is the widespread use of antibiotics in the poultry industry that is the main risk factor for an increase in the occurrence of bacterial resistant strains. Bacteria display variable levels of resistance to antibiotics. The high incidence of antibiotic resistance among the bacteria populating poultry and rising frequency of the bacterial strains represent a public health hazard. Applications of antibiotics in poultry production bring about an increase in resistance to antibiotics not only in pathogenic bacterial strains, but also in commensal bacteria (Lukasova and Sustackova, 2003). In this respect, gastro-intestinal commensal bacteria constitute a reservoir of resistance genes for pathogenic bacteria. Their level of resistance is considered to be a good indicator for selection pressure for antibiotic use and for resistance problem to be expected in pathogens. Poultry products and meat are a common reservoir of emerging antibiotic resistances available to bacteria inhabiting humans. It can be supposed that the transmission of antibiotic-resistant bacteria to people who got in contact with these sources through direct ingestion or handling results in an increase in the human reservoir of these strains which can rapidly spread to the community. In theory, the birds’ waste may serve as a vehicle for expanding the transmission of resistance bacteria to humans. In this direction, the waste ends up in water mainly wells and ponds which represent a significant source of natural water supply for rural population in the developing countries. Using hatchery waste from poultry where antibiotics have been used uncontrolled will further compound the problem of antibiotic resistance in the aquaculture industry.

This study sets out to identify the bacterial flora from the skin and digestive system of cultured catfish fed with poultry hatchery waste and also to evaluate and present the results of the antibiotic sensitivity test of the isolated bacterial flora from catfish fed with poultry hatchery wastes.

**MATERIALS AND METHODS**

**Study Location**

The study was conducted in five purposively selected fish farms and three commercial poultry hatcheries in Ibadan. The Poultry hatcheries were all located in Oluyole Industrial Estate in Ibadan Southwest Local Government of Oyo State while the fish farms were located in Ido, Oluyole and Southwest Local Government Areas of Ibadan, Oyo state, Nigeria. The choice of the study locations was based on their accessibility and owners’ permit on the use of facilities.

**Collection and Processing of Samples**

15 samples of hatchery wastes were collected in sterile polythene bags immediately after hatching.
the chicken eggs. The samples were collected for a period of two weeks. 60 tissue samples (20 each from skin, stomach and intestines) were harvested aseptically from a total of 20 live catfish collected from the five selected catfish farms. All the samples were then transported in a cooler box containing ice packs to the Food and Meat Hygiene Laboratory of the Department of Veterinary Public Health and Preventive Medicine, University of Ibadan and Department of Microbiology and Parasitology, University College Hospital (UCH), Ibadan, Oyo state.

1 cm³ of skin and 1g portion of stomach and Intestine were cut and weighed aseptically from each of the catfish tissue samples and 1g from each of the hatchery waste samples was aseptically weighed and taken for bacteriological examinations. Each of these samples was homogenized in 9ml peptone water that had been prepared aseptically according to manufacturer’s instruction. 3-folds and 2-folds serial dilution of each homogenised sample from hatchery waste and fish tissues were further carried out respectively.

Bacteriological Examination of the Samples

Inoculation of diluted samples – 0.1ml of the desired solution of the sample was taken and inoculated (surface spread technic) aseptically unto already prepared cultured media namely; Nutrient agar, Eosin-methylene blue (EMB) agar, MacConkey agar and Salmonella-shigella agar (SSA). All the culture media were all prepared aseptically according to the manufacturer’s instruction. The inoculated media plates were then incubated for between 18- 24 hours at 37˚C. After incubation, distinct bacterial colonies were counted to determine the colony forming unit (CFU) per gram of the sample (Horsely, 1977, APHA, 1995).

Sub-culturing of isolates- distinct colonies were further subcultured on freshly prepared culture media; Nutrient agar, MacConkey, EMB agar and SS agar to obtain pure isolates of the organisms.

Identification of Pure isolates; after isolation of the pure colonies, the isolates were further identified morphologically and biochemically (Baron and Murray, 1999), using gram staining technique and Microbact Identification Kits (Microbact™ GNB 12A/B/E,24E, Oxoid ). Microbact™ Gram-Negative Identification Kits- it is designed to simulate conventional biochemical substrates used for identification of Enterobacteriaceae and common miscellaneous Gram-negative bacilli. Organism identification is based on pH change and substrates utilization (Cowan, 1977 and Farmer, et al, 1985).

ANTIBIOTICS SENSITIVITY TEST OF THE ISOLATED BACTERIAL ORGANISMS

The test bacterial isolates were inoculated unto nutrient agar and followed by application of the discs (oxoid Ltd) impregnated with different antibiotics. Agar disc diffusion method (Baur et al., 1996; SFM, 2003) was employed. Antibiotic discs contained the following seven antibiotics Nitrofurantoin (F) -300μg, Augmentin( AMC)- 30μg , Ciprofloxacin (CIP)- 5μg, Nalidixic acid (NA)- 30μg, Erythromycine (E)- 10μg, Chloramphenicol (C)- 30μg, Gentamicine (CN)-10μg.


Data entry and analysis was done using SPSS version 15. Both descriptive and inferential statistics were used. The mean total bacterial and enterobacterial counts in the hatchery waste samples and fish organs (skin, stomach, & intestine) were calculated. One-way analysis of variance was used to compare means among the three different hatcheries and organs. The level of significance was set as p < 0.05.

Results.

Table 1.0 shows the bacterial isolates obtained from the different catfish organs and the hatchery waste. The species of the bacterial isolated are those in the genera Citrobacter, Esherichia, Staphylococcus, Pseudomonas, Salmonella, Bacillus, Leclercia and Klebsiella.

All the samples of the hatchery waste were contaminated with the microbial load (log_{10} CFU/g) in the range of 5.28-5.66. The enterobacterial load (log_{10} CFU/g) of the hatchery waste samples was in the range of 4.30-5.48.

The organs were contaminated with bacteria load (log_{10} CFU/g or cm²) in the range of 5.08-5.75. The enterobacterial load (log_{10} CFU/g or cm²) was in the range of 4.06-5.66 with skin having the lowest. The result of total bacterial count of the fish are presented in table 2 while that of enterobateriaceae count in the skin, stomach and small intestine are presented in table 3. The zones of inhibition of the isolates from hatchery waste and the different organs in the catfish are as presented in tables 4 and 5 respectively.
Table 1. Bacterial isolates obtained from different catfish organs and hatchery waste

<table>
<thead>
<tr>
<th>SAMPLES</th>
<th>BACTERIA ISOLATES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>Citrobacter spp, Eschericia coli, Staphylococcus aureus, Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>Stomach</td>
<td>Salmonella subspp1, Leclercia adecarboxylata, Bacillus spp, Klebsiella pneumonia, Eschericia coli, Staphylococcus aureus</td>
</tr>
<tr>
<td>Intestine</td>
<td>Eschericia coli, Salmonella arizonae subspp34</td>
</tr>
<tr>
<td>Hatchery waste</td>
<td>Pseudomonas aeruginosa, Eschericia coli, Staphylococcus epidermidis, Bacillus spp, Klebsiella pneumonia,</td>
</tr>
</tbody>
</table>

Table 2.0: Mean total bacterial load count in selected organs of cat fish fed hatchery waste.

<table>
<thead>
<tr>
<th>Organs</th>
<th>Mean ± SD (n = 20)</th>
<th>F-value</th>
<th>P-value</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>33.1 ± 9.0</td>
<td>2.02</td>
<td>0.14</td>
<td>Not Significant</td>
</tr>
<tr>
<td>Stomach</td>
<td>37.1 ± 7.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestine</td>
<td>32.7 ± 6.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.0: Mean enterobacteria load count in selected organs of cat fish fed hatchery waste.

<table>
<thead>
<tr>
<th>Organs</th>
<th>Mean ± SD (n = 20)</th>
<th>F-value</th>
<th>P-value</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>17.5 ± 8.6</td>
<td>1.38</td>
<td>0.26</td>
<td>Not Significant</td>
</tr>
<tr>
<td>Stomach</td>
<td>14.1 ± 9.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestine</td>
<td>13.3 ± 8.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.0 (a): Diameter (mm) of zones of inhibition to individual antibiotics.

<table>
<thead>
<tr>
<th>Isolates from Poultry Hatchery samples</th>
<th>F(300μg)</th>
<th>NA(30μg)</th>
<th>CIP(5 μg)</th>
<th>C(30 μg)</th>
<th>E(10μg)</th>
<th>AMC(30μg)</th>
<th>CN(10 μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>20</td>
<td>14</td>
<td>23</td>
<td>2</td>
<td>-</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>-</td>
<td>-</td>
<td>25</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus spp</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>8</td>
<td>17</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>14</td>
<td>21</td>
</tr>
</tbody>
</table>

F- Nitrofuratoin, AMC – Augmentin, CIP – Ciprofloxacin, NA - Nalidixic acid, E- Erythromycine, C – Chloramphenicol and CN – Gentamicine.

Table 5.0 (b): Diameter (mm) of zones of inhibition to individual antibiotics.

<table>
<thead>
<tr>
<th>Isolates from Catfish samples</th>
<th>F(300μg)</th>
<th>NA(30μg)</th>
<th>CIP(5 μg)</th>
<th>C(30 μg)</th>
<th>E(10μg)</th>
<th>AMC(30μg)</th>
<th>CN(10 μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>20</td>
<td>15</td>
<td>25</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Salmonella arizonae subspp3</td>
<td>18</td>
<td>15</td>
<td>25</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Escherichia fergusonii</td>
<td>18</td>
<td>12</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>16</td>
<td>-</td>
<td>25</td>
<td>-</td>
<td>-</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>Leclercia adecarboxylata</td>
<td>18</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Salmonella subspp1</td>
<td>-</td>
<td>20</td>
<td>26</td>
<td>-</td>
<td>-</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>Bacillus spp</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>


DISCUSSION

In the aquaculture industry, resistance to antibiotics has been commonly reported (for example, Rhodes et al. 2000; Schmidt et al. 2001; Kim et al. 2004; Hatha et al. 2005; Akinbowale 2006 and Adedeji et al 2011) all reported the resistance of different bacteria to different antibiotics in their studies. According to Apatasi (2009) it is the widespread use of antibiotics in the poultry industry that is the main risk factor for an increase in the occurrence of bacterial resistant strains. Bacteria display variable levels of resistance to antibiotics.
The high incidence of antibiotic resistance among the bacteria populating poultry and rising frequency of the bacterial strains represent a public health hazard. Applications of antibiotics in poultry production bring about an increase in resistance to antibiotics not only in pathogenic bacterial strains, but also in commensal bacteria (Lukasova and Sustackova, 2003). In this respect, gastro-intestinal commensal bacteria constitute a reservoir of resistance genes for pathogenic bacteria. Their level of resistance is considered to be a good indicator for selection pressure for antibiotic use and for resistance problem to be expected in pathogens. Poultry meat, products and waste are common reservoir of emerging antibiotic resistances available to bacteria inhabiting humans using hatchery waste from poultry where antibiotics have been use uncontrolled will further compound the problem of antibiotic resistance in the aquaculture industry.

In this study, the species of bacteria isolated are those in the genera Citrobacter, Escherichia, Staphylococcus, Pseudomonas, Salmonella, Bacillus, Leclercia, Klebsiella. The bacterial load observed in the hatchery waste was in the range of $1.9 \times 10^5$ to $4.6 \times 10^5$. The total number of bacterial count for fresh catfish ($Clarias gariepinus$) was in the of $1.2 \times 10^6$ cfu/g and $5.6 \times 10^5$ of fish organs, and this number fell within accepted limit according to Anon (1991) who said that the acceptability limit is $10^6$ cfu/g for mesophilic aerobic bacteria. Also Sikorski (1990) reported that for high quality fresh fish, the number of bacteria present on the surface vary from $10^3$ – $10^5$ cfu/g. These results agree with finding of Chou (1993) who reported that the total aerobic plate count of unwashed and washed catfish frame mince without cryoprotectants were $5 \times 10^6$ cfu/g and 106 cfu/g, respectively.

However, in spite of the normal bacterial load seen in this study, bacterial organisms isolated are pathogenic and capable of causing diseases in both fishes and humans under poor hygiene practice and in immuno-compromised individual and animals. This study has also shown that the enteric bacteria isolated from hatchery waste and fish were resistant to some antibiotics. $E$ coli was sensitive to all the antibiotics except Erythromycin. $Salmonella$ arizonae subsp.3 was also sensitive to all the antibiotics except Nalixidic acid, Erythromycin, and Augmentin. Erythromycin was not active against any of the isolated organisms while $Bacillus$ spp was not sensitive to any of the antibiotics. In this study therefore, a multidrug resistance pattern was observed in most of the bacterial isolates. However, the bacterial species were susceptible to the antibiotics Ciprofloxacin, followed by Gentamycin, Augmentin and Nitrofuratoain in that order. Resistance to Erythromycin was wide spread in this study. Resistance to Erythromycin might be related to their overuse. Secondly, resistance of Gram-negative bacteria to erythromycin is to be expected because of intrinsic resistance of many such organisms to macrolide antibiotics. The emergence and dissemination of multiple antimicrobial resistance among $E$.coli, $Salmonella$ spp is an increasing global health problem that is complicating the therapeutic management of severe salmonellosis and diarrhogenic diseases.

The variety of resistance pattern observed in this study can be correlated to antibiotic use in Nigeria and are similar from those seen in countries where antibiotics are known to be used in aquaculture. Chloramphenicol resistance has also been reported in Chile (Mirand and Zemelman 2002), France (Michel et al., 2003) and in a Western Mediterranean study (Chelossi et al. 2003). Quinolone resistance has been reported in environmental isolates at a relatively low frequency (McKoen et al. 1995; Guardabassi et al. 1998); however, Chelossi et al. (2003) reported resistance to nalidixic acid in 70% of their isolates. The result showed resistance to more than one class of antibiotic. Multiple drug resistance has been reported in a number of studies of fish pathogens and aquaculture environments (McPhearson et al. 1991; Schmidt et al. 2000; Hatha et al. 2005; Adedeji et al., 2011).

Tjahjadi et al., 1994, Miranda and Zemelman (2002) reported that bacteria resistant to six to ten antibacterials were common. Studies on the antibiotic resistance in bacteria from shrimp ponds (Tendencia and de la Pena 2001) demonstrated a correlation between multiple bacterial antibiotic resistance levels and use of particular drugs. The types of bacterial organisms that are associated with the hatchery waste and catfish fed hatchery waste found in this study call for concern.

In conclusion, the widespread use of antibiotics in fish farming for therapeutic, prophylactic purposes and as growth promoters can promote the emergence of resistance in bacteria. Many of the antibiotics investigated in the present study are known to be used in veterinary practice and the poultry industry. It is therefore necessary to monitor the usage of antibiotics in aquaculture practices to prevent the frequency of development of resistant fish pathogens and bacteria. Moreover the transferability of fish pathogens that are multi-drug resistant through horizontal gene transfer in the food chain has human health consequences (Nawaz et al., 2001; Heuer et al., 2009).
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