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# Prevalence, antibiograms and plasmid profile of *Campylobacter* species from animal faeces in Ondo State, Nigeria.

**Mensah-Agyei, G. O.*; Osisami, O. F. And Ezeamagu, C. O.**

*Department of Microbiology, School of Science and Technology, Babcock University, Ogun State, Nigeria*

Corresponding author < mensah-agyeig@babcock.edu.ng >

## Abstract

Bacteria strains can be transferred from animal to humans by direct or indirect contact with animal faeces, meat and other products. With the emergence of resistant strains, such animal product poses threat to the public health. *Campylobacter* species are major foodborne pathogens associated with human gastroenteritis and they can also contaminate animal foods. However, there is paucity of information on this species from animal sources in South-West Nigeria. Therefore, this study aimed to determine the prevalence, antibiogram and plasmid profile of *Campylobacter* species in animal wastes in Ondo state. One hundred and fifty faecal samples were collected from cow, goat, poultry, fish and pig and subjected to conventional microbiological analyses. The isolates were screened against eight antibiotics and the plasmid profiles of resistant strains were determined. Fifty isolates of *Campylobacter* were recovered from the animal wastes with cow dung and poultry dropping having higher percentage of the recovered species (30% and 28% respectively). Gentamicin, Augmentin, Nitrofurantoin and Ampicillin recorded 100% resistance each followed by Ceftazidime (90%), Cefuroxime (86%), Ciprofloxacin (84%) and Ofloxacin (78%). Multi-drug resistance (MDR) was also observed as all the isolates showed resistance to at least three antimicrobial classes. The high resistivity of *Campylobacter* species raises huge concern regarding public health. It is, therefore, necessary to set up antibiotic use surveillance system in animal (agricultural) practice to prevent increase in antibiotic resistance among *Campylobacter* species.

**Keywords:** Animal faecal waste, antibiogram, *Campylobacter* species, gene transfer, prevalence.

**Introduction**

*Campylobacter* species are curved, S-shaped, non-spore forming Gram-negative bacteria, of the family *Campylobacteraceae*, that require little or no oxygen supply for growth (Ghane et al., 2010). The *Campylobacter* genus consists of an average of 17 species and 6 subspecies. This genus is made up of organisms with complex nutritional requirements (fastidious organisms) (Man, 2011). *Campylobacter* causes diseases in animals and humans (O'Brien, 2017). Most species of animal including cat, cattle, chicken, dog, pig and turkey is susceptible to infection by *Campylobacter* (Sahin et al., 2017). Animals can be exposed to the *Campylobacter* species bacteria by direct contact with infected animals, or through contaminated feed or water. Raw or undercooked meat fed to pets may also contain the *Campylobacter* species.

Being a zoonotic pathogen, infections can be transferred from animals to humans through contaminated food, water, milk and animal droppings from the environment. *Campylobacter* spp. can also be transmitted through the faecal-oral route as well as direct contact with an infected animal. (Ellis-Iversen, 2012). The main reservoirs for this organism are the poultry birds. *Campylobacter* species, including *C. coli* and *C. jejuni*, are implicated in human diseases like the bacterial gastroenteritis (Moore et al., 2005) and in fact, a leading cause of acute gastroenteritis worldwide (Fitzgerald, 2015). Clinical manifestation of *campylobacter* infection includes diarrhea, abdominal pain, fever and vomiting. In infants and young children, infection causes moderate to severe diarrhea. The highest incidence rate of *Campylobacter* species infection is among children below the age of two (Pan et al., 2008).

*Campylobacter* produces two types of toxins; cytotoxins and enterotoxins. The cytotoxins are responsible for bloody diarrhoea, while the enterotoxins are similar to *Escherichia coli* heat-liable toxin and those produced by *Vibrio cholerae. Campylobacter coli* produce enterotoxins to a lesser degree (Hernández-Cortez et al., 2017).

The first line antimicrobial treatment for *Campylobacter* infection are macrolides, tetracycline and fluoroquinolones. Although, most *Campylobacter* infection are self-limiting and do not require therapy, replacement of fluids and electrolytes lost through diarrhea is critical for infected individuals. But in a severe case and where infected individuals are immunocompromised or have complications such as septicemia, treatment with antibiotics may be required (Kim et al., 2010). The indiscriminate use of antibiotics in veterinary and animal husbandry has led to the development and increase of antibiotic resistance in the treatment of *Campylobacter* infection (Chang et al., 2017). This has resulted in increased chances of treatment failure as well as increase in the cost of treatment.

Recently, the Centres for Disease Control and Prevention (CDC) recorded drug-resistant *Campylobacter* as a huge threat in the United States. Resistance of *Campylobacter* to antibiotic agents is interceded by multiple mechanisms (Su et al., 2017). The resistance mechanisms of the *Campylobacter* species bacteria include horizontal gene transfer (HGT) or multidrug efflux pump (Su et al., 2017). One of the most studied genes that confer resistance on *Campylobacter* is tet(O) gene, a ribosomal protection protein that is carried on self-transmissible plasmids in both *C. coli* and *C. jejuni* (Abdi-Hachesoo et al., 2014). This gene binds to the bacterial ribosome and displaces tetracycline (Connel et al., 2003). The tet(O) gene can be located on both plasmids and chromosomes, and is associated with high levels of resistance (Pratt & Korolik, 2005).

There is a growing concern on the transmission of resistant bacteria via the food chain. The use of antibiotics as a dietary supplement in livestock farming has been stated as a leading factor in the emergence of antibiotic resistant strains (Bottemiller, 2013). This inappropriate use of antibiotics in livestock farming causes a change in the plasmid properties, which could in turn lead to an increase in pathogenicity, virulence and antibiotic resistance; thus, posing a direct threat to the environment and public health. Therefore, the knowledge of the antibiotic susceptibility patterns and plasmid profile of *Campylobacter* species in environmental samples will be useful in effective antibiotic surveillance and food safety monitoring. This present study was therefore aimed at evaluating the resistance pattern and plasmid profiles of *Campylobacter* species isolated from environmental samples in Ondo state of Nigeria.

## Materials and Methods

**Sample collection:** One hundred and fifty animal faecal waste samples were obtained from Ondo State in January 2019 for the present study. The distribution of samples was: 18 goat dung, 40 poultry bird droppings, 11 pig dung, 32 fish pond water and 49 cow dung. Samples were collected in clean Ziplock bags. All collected samples were immediately transported in an ice box to the Microbiology laboratory, Babcock University and processed on the same day of sample collection.

**Sample processing:** Each sample (10g) was diluted in 90ml of sterile normal saline, homogenized and diluted ten-fold. Aliquots (1 ml) of the diluted samples were surface-plated on freshly prepared Chromogenic agar (Oxoid Ltd., Basingstoke, Hampshire, England) supplemented with 5% defibrinated horse blood and selective supplements (5ml Cycloheximide, 3mg Rifampicin, 2ml Polymyxin B, 5mg Trimethoprim). All the inoculated plates were incubated at 37 $^{o}$C for 24–48 h under microaerophilic conditions simulated in a candle jar. Colonies suspected to be those of *Campylobacter* based on their typical cultural characteristics or colony morphology were subjected to further tests including Gram staining, catalase and oxidase tests (PHE, 2018). Isolated *Campylobacter* strains were preserved in Nutrient broth containing 45% glycerol at -50$^{o}$C and were subsequently cultured on an appropriate medium supplemented with 5% horse blood when required**.**

**Antimicrobial Susceptibility Testing:** Revived cultures from glycerol stock were inoculated into nutrient broth overnight at 42$^{0}$C under microaerophilic condition and the turbidity of the bacterial suspension was adjusted to 0.5 McFarland standard. Using a sterile swab stick, the culture was swabbed uniformly onto an already prepared Mueller Hinton agar plate. The standard Kirby-Bauer disk diffusion method (Bauer et al., 1966) was employed in conducting the antimicrobial susceptibility test using antibiotic impregnated disks: ceftazidime30µg, cefuroxime 30µg, gentamicin 10µg, ampicillin 10µg, ofloxacin 5µg, augmentin (Amoxicillin/Clavulanic acid) 30µg, nitrofurantion 300µg and ciprofloxacin 5µg. The plates were then incubated at 37$^{0}$C for 24 h and observed for zones of inhibition measured in mm. Susceptibility categorization [susceptible (S), intermediate (I) and resistant (R)] was performed according to the Clinical and Laboratory Standards Institute using the Enterobacteriaceae family breakpoints for result interpretation (CLSI, 2012).

Multidrug resistance (MDR) was defined as resistance to three or more antimicrobial classes (Ezeamagu et al., 2017). The class definitions used were aminoglycosides (gentamicin), Cephalosporin (ceftazidime, cefuroxime), β-lactam (ampicillin, Amoxicillin/Clavulanic acid), Nitrofuran (nitrofurantoin) Quinolone (ciprofloxacin, ofloxacin) (Willey et al., 2011).

**Plasmid Isolation:** Plasmid DNA was extracted using the Mini Prep method of Lech and Brent (1987) as modified by Ezeamagu et al. (2017). Whole colony from Luria-Bertani medium (LB agar) plate pre-treated with antibiotics was resuspended in 5ml LB (pre-treated with antibiotics selective for the bacterium). The inoculated broth was incubated at 37$^{o}$C overnight with gentle shaking at 70rpm. The overnight bacterial suspension (1.5ml) was dispensed into an Eppendorf tube and centrifuged at 13,000rpm for 1 min. The supernatant was decanted into a new Eppendorf tube and vortexed. TENS solution (Tris 25mM, EDTA 10Mm, NaOH 0.1 N and SDS 0.5%; 300µl) was added and mixed by inversion for about 5 min until the solution became sticky. Thereafter, 150µl of 3.0 M sodium acetate (pH 5.2) was added, vortexed and then centrifuged for 5 min to pellet cell debris and chromosomal DNA. The supernatant was transferred to another Eppendorf tube, 900µl of ice-cold absolute ethanol was added and the mixture was centrifuged for 10 min to pellet plasmid DNA. The supernatant was discarded while the pellet was washed in two changes of 1ml of 70% ethanol prior to drying. The dried pellet was re-suspended in 40µl of distilled water. The extracted plasmid (10µl) was resolved by 0.8% agarose gel electrophoresis.

## Results

A total of 50 *Campylobacter* isolates were recovered from the 150 samples processed. Three *Campylobacte*r isolates (prevalence: 6%) were obtained from goat dung, 14 (prevalence: 28%) from poultry bird droppings, 9 isolates (prevalence: 18%) from pig dung, 9 isolates (prevalence: 18%) from fish pond water samples and 15 isolates (prevalence: 30%) from cow dung (Table 1).

Table 2 shows the antimicrobial sensitivity pattern of *Campylobacter* species that were isolated in this study. All 50 (100%) isolates were resistant to gentamicin, augmentin (amoxicillin/Clavulanic acid), nitrofurantoin and ampicillin, while 43 (86%), 42 (84%) and 39 (78%) showed resistance to cefuroxime, ciprofloxacin and ofloxacin, respectively. About 6(12%), 2(4%) and 1(2%) of the *Campylobacter* isolates were sensitive to ofloxacin, ceftazidime and ciprofloxacin, respectively. Furthermore, 7 (14%) isolates recorded intermediate susceptibility to cefuroxime and ciprofloxacin while 1(2%) isolate recorded intermediate susceptibility to ceftazidime.

All the isolates showed resistance to not less than four antibiotics (Table 2). It was observed that 62% of the isolates resisted all the eight antibiotics used in this study (Figure 1). Moreover, 26% of the total isolates were resistant to 4 different combinations of 7 antibiotics, while 8% of the isolates were resistant to a combination of 6 antibiotics and 4% to a combination of 4 antibiotics (Fig. 1). Plasmid profiling showed that 53% of the 15 randomly selected multi-drug resistant isolates possessed plasmids of size 21.13kb, which conferred the antibiotic resistant characteristics of the carrying organism (Fig. 2).

TABLE 1: Prevalence of *Campylobacter* isolates from various sources

| Sources | Number of Samples Obtained | Number of Isolates | Prevalence (%) |
|---|---|---|---|
| Goat faecal waste | 18 | 3 | 6 |
| Poultry dropping | 40 | 14 | 28 |
| Pig faecal waste | 11 | 9 | 18 |
| Fish pond water | 32 | 9 | 18 |
| Cow faecal waste | 49 | 15 | 30 |
| | 150 | 50 | |

TABLE 2: Susceptibility pattern of *Campylobacter* species isolated from environmental samples

| ANTIBIOTICS | SENSITIVITY | | INTERMIDIATE | | RESISTIVITY | |
|---|---|---|---|---|---|---|
| | NO OF ISOLATE | PERCENTAGE | NO OF ISOLATE | PERCENTAGE | NO OF ISOLATE | PERCENTAGE |
| Ceftazidime (30µg) | 2 | 4 | 1 | 2 | 47 | 90 |
| Cefuroxime (30µg) | 0 | 0 | 7 | 14 | 43 | 86 |
| Gentamicin (10µg) | 0 | 0 | 0 | 0 | 50 | 100 |
| Ciprofloxacin (5µg) | 1 | 2 | 7 | 14 | 42 | 84 |
| Ofloxacin (5µg) | 6 | 12 | 5 | 10 | 39 | 78 |
| Augmentin (amoxicillin/Clavulanic acid) (30µg) | 0 | 0 | 0 | 0 | 50 | 100 |
| Nitrofurantoin (300µg) | 0 | 0 | 0 | 0 | 50 | 100 |
| Ampicillin (5µg) | 0 | 0 | 0 | 0 | 50 | 100 |

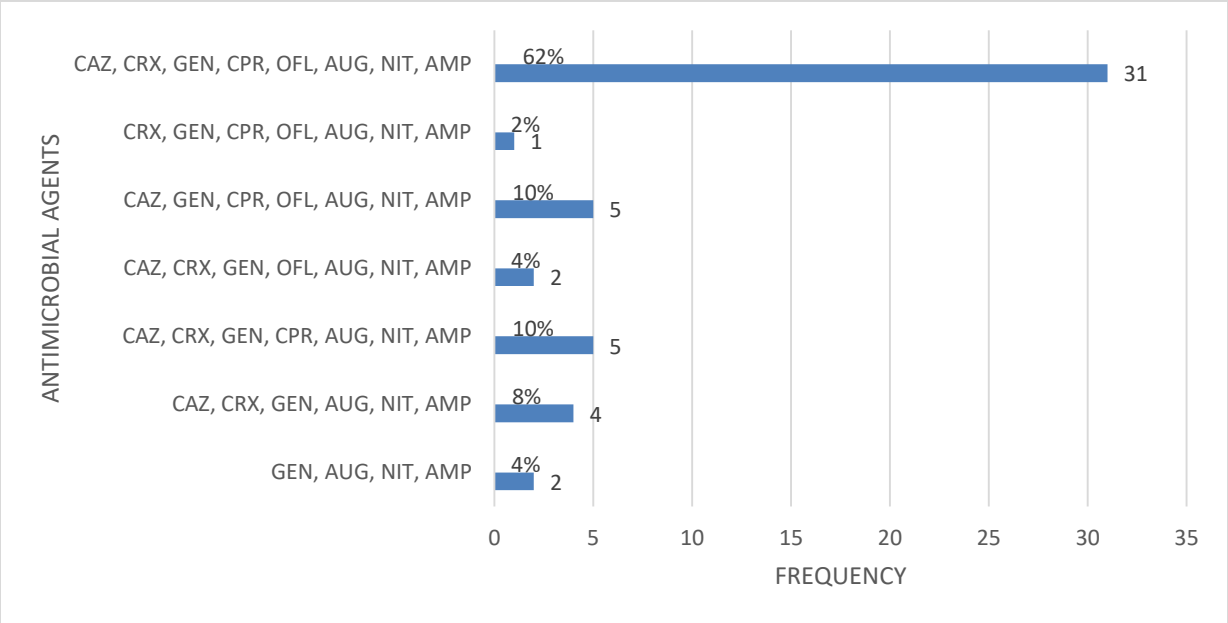


FIGURE 1: Multi-Drug Resistance Pattern Among Isolates.

KEYS: CPR=Ciprofloxacin; NIT=Nitrofurantoin; CAZ=Ceftazidime; GEN=Gentamicin; CRZ=Cefuroxime; AUG=Augmentin; OFL=Ofloxacin; AMP=Ampicillin


Figure 2: Gel picture of isolates with plasmid

M: Molecualr ladder, Lane 3, 5, 6, 10, 11, 13, 14, 15- isolates with plasmid

**Discussion**

A total of 50 *Campylobacter* isolates were obtained in the present study. The prevalence according to the sample/source ranged from as low as 6% in goat dung to as high as 30% in cow dung while poultry droppings, fish pond water and pig dung had less than 70% prevalence. The high prevalence of *Campylobacter* isolates from pig dung was similar to a study previously reported by Burrougha et al. (2013) and Varela et al. (2007). Similarly, the prevalence of *Campylobacter* isolates from poultry in this study agreed with that reported by Luu et al. (2006).

This present study shows that there is a high resistivity of the *Campylobacter* isolates to the tested antibiotics. More so, all the isolated strains showed multiple antibiotics resistance due to the exhibited resistance to four different antibiotics. Specifically, there was a 100% resistance to ampicillin, augmentin, gentamicin and nitrofurantoin. The high resistance rate to gentamicin in this study is consistent with the reports of Olatoye and Ogunsemoyin (2016) for 100% resistance and similar to the findings of

Ezeamagu et al. (2017) who recorded 73% resistance; both studies were carried out in Oyo State. However, our findings contrast that of Ghimire et al. (2014) who recorded 5.56% resistance to gentamicin at Chitwan, Nepal.

Resistance to quinolones was 84% for ciprofloxacin and 78% for ofloxacin. This is similar to the findings of Kim et al. (2019) whose study indicates an 85.6% resistance to ciprofloxacin, but higher than the 57.8% resistance reported by Kim et al. (2010). The disparity in the results may be due to the low number of samples reported in the previous study. Fluoroquinolones and macrolides are choice drugs for the treatment of campylobacteriosis (Wieczorek & Osek, 2013). The effectiveness of aminoglycosides in treating human *Campylobacter* infections have as well been reported (Wieczorek & Osek, 2013). High resistance of *Campylobacter* to these drugs as observed in the present study as well as in previous reports may be an indication of a looming danger to humans and further limiting of effective therapeutic options, especially when such resistant strains are transferred to the human population.

β-lactams are were also tested on the isolates in this study and there was 100% resistance to the two tested antibiotics of the β-lactam class. Premarathne et al. (2017) and Ghimire et al. (2014) also obtained a high resistance (69.2% and 92.6%, respectively). A major β-lactamase, $bla_{OXA-61}$ gene, which is responsible for β-lactamase-mediated β-lactam resistance (Proietti et al., 2020), is prevalent among *Campylobacter* spp. of veterinary origin. The Production of $bla_{OXA-61}$ is associated with resistance to penams (Griggs et al., 2009).

This study shows a high level of resistance of *Campylobacter* isolates to commonly used antibiotics in the health sector. The comparison of this present study to those that have been reported in the past shows increasing antibiotics resistance in *Campylobacter* species and this should raise concern because of the possible transfer of resistant *Campylobacter* strains from animal to human through the food chain. Campylobacteriosis in humans may occur via direct ingestion of *Campylobacter*-contaminated (or undercooked) food, or by cross-contamination from contaminated raw meat to ready-to-eat foods via unwashed hands or contaminated kitchen utensils (Park et al., 1991).

Human infection with antibiotic-resistant bacteria has been associated with increased morbidity and treatment failure. Large amounts of antibiotic agents are used in modern animal farming as prophylactics, to increase productivity and as growth promoters (Bengtsson & Greko, 2014). These actions may provide an enabling environment for the spread and persistence of antibiotic-resistant zoonotic bacteria such as *Campylobacter*. Globally, the use of antimicrobials in food animals was estimated at 63,000 tons annually and is projected to increase by almost 70% in the year 2030 (Alhaji & Isola, 2018). Besides the top users which are China, US and Brazil; the largest relative increase is projected to take place in the developing countries with Myanmar, Indonesia and Nigeria taking the lead among those that may experience over 200% increase (Alhaji & Isola, 2018). Therefore, antibiotic susceptibility patterns among *Campylobacter* strains isolated from food production animals must be monitored as an important component of the risk assessment of antibiotic use in agriculture (Engberg et al., 2000).

The plasmids carrying resistance determinants are transferrable and may be passed between bacteria through conjugation (Lerminiaux & Cameron, 2019). Here, we found plasmids similar to the size 21.13kb; this was also previously reported by Ezeamagu et al.

(2017) for plasmid that harbours resistance determinant to β-lactam antibiotics. Zoonotic bacteria can be multi-drug resistant environmental determinants if they are transferred through media like water, plant and animal feed and faeces. Therefore, it is of huge importance that resistance among environmental bacteria be monitored, as well as the animals and their products intended for human consumption. More often than not, the birth of resistant bacteria population has been linked to the use, over-use and abuse of antibiotics. Once resistance is developed, it is transferred to other bacterial pathogens by horizontal gene transfer with the aid of genetic elements such as plasmids, transposons and integrons (D'Costa et al., 2011).

In this research, it has been established that the resistance factor may not necessarily be due to the feeds, they could also be from the environment (such as animal faecal waste). Humans and animals can acquire infections with bacteria carrying drug resistant plasmids when they come in contact with these bacteria in the environment. Under favourable physical, chemical and biological conditions, exchange of plasmids will occur. The drug resistant strains from the environment can then spread to man and other animal population, therefore, it is of utmost importance that antibiotic use in animal husbandry be monitored adequately and to establish a proper animal waste management.

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