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Association of Plasmid Bearing Multi Drug Resistant Bacteria with High Mortalities in Nigerian Poultry

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Abstract
Bacteriological investigations were carried out on reported cases of low and high mortalities in commercial poultry in the year 2007-2009 in commercial flocks of breeders, layers, broilers, chicks and growers. Pure bacterial isolates obtained from dead birds, mainly Gram negative, were tested for their biochemical and antibiotic susceptibility profiles using Microscan® Dried Gram-negative Breakpoint Combo Pannels. The microscan panel analysis bacterial isolates for 24 biochemical tests and up to 25 antimicrobial agents following 16-20 hours of incubation at 35-37^{o}C. Bacteria isolated were *Escherichia coli*, *Salmonella* Enteritidis, *Salmonella* Pullorum, *Salmonella* Gallinarum, *Citrobacter youngae, Klebsiella ozaneae, Klebsiella variicola, Enterobacter cloaceae, Hafnia alvei, Pasteurella gallinarum and Pasteurella multocida*. The bacterial isolates showed resistance to 6-23 of the 25 antibiotics tested. Tetracycline and ampicilllin were found as the least potent drugs, with 90 and 90.91% resistance respectively. Resistance was obtained against antibacterial agents that are not in use in Nigerian poultry industry, such as the cephalosporins, carbapenems, piperacillin, tircacillin, amikacin, tobramcycin and levofloxacin suggesting possible genetic contribution to resistance. Thirty-four (85%) of the 40 pathogenic bacteria isolates treated for plasmid profile were found to be carrying plasmids. Plasmid DNA size 11.50 kbp was commonly observed (about 65%) among all the plasmid borne mdr bacteria, plasmid DNA size 10kbp (incidence of about 22% in plasmid borne bacteria) as well as a 26.3 kbp plasmid DNA and a 6.6 kbp plasmid DNA. The susceptibility of the plasmid bearing bacteria to antibiotics was enhanced following plasmid curing with sodium deodecyl sulphate. Antibiotic susceptibility testing should guide treatment in all infections where organism can be cultured in veterinary medicine practice
Keywords: Antibiotics, high mortalities, multi-drug resistant bacteria, plasmid, poultry.

1.0 Introduction
Intensive animal production is associated with large scale antimicrobial use worldwide (Moulin, 2001). This often leads to a high level of colonization of animals with anti-microbial resistant bacteria (Aubry-Damon *et al.,* 2004). Infections due to such drug resistant organisms are of grave consequences as they can lead to high economic lose in poultry (Poppe *et al.,*1991). Multidrug resistant gram-negative bacteria have been emerging worldwide(Yamane *et al*., 2005) and resistance to antibiotics could be plasmid or chromosomal mediated (Mayer, 1988). Diagnostic uncertainty has however been reported as a key driver of drug misuse and overuse, which can lead to antimicrobial selection pressure and increased rates of resistant microbes (Nuermberger and Bishai, 2004).
In Nigeria, small scale and back-yard poultry farms are many and may contribute close to fifty percent of total poultry production in the country (Obayelu, 2007). The owners do manage the health on their own or rely on quacks, but go for veterinarians only when conditions go beyond control. Multi-drug resistant bacteria have been found to be associated with drop in egg production and high mortalities in Nigeria poultry industry, resulting in heavy economic losses (Okiki and Ogbimi, 2008, 2010a,b). The aim of this study was to describe the antibiotic resistant pattern and its probable genetic role, of bacteria causing high mortality in Nigerian poultry industry.

2.0 Materials and Methods
Investigations were carried out on the possibility of bacteriological cause of reported cases of low and high mortalities in commercial poultry, in the year 2007-2009, in commercial flocks of breeders (7), layers (30), broilers (27), chicks (20) and growers (18) in Lagos and Ogun States of Nigeria.
2.1. *Bacteriological Investigation*
A loopful of fluid from the lung, liver and heart blood of dead birds was streaked separately on freshly prepared nutrient agar plates. Plates were incubated at 37^{o}C for 18-24 hours. Discrete colonies of bacteria were subcultured to obtain pure cultures. Isolates were gram stained and morphology obtained by microscopy. Isolates were cultured on nutrient agar slopes and kept at 4^{o}C till use.
Characterization of bacterial isolates was carried out using the Microscan® Dried Gram-negative Break-point

Combo Panels (Dade Behring, USA). MicroScan® panels are designed for use in determining antimicrobial susceptibility and identification to species level of aerobic and facultative anaerobic Gram-negative bacilli. The system analysis bacterial isolates for 23 – 24 biochemical test and susceptibility to 25 antimicrobial agents within 16 – 20 hours of incubation at 35 – 37°C. The MicroScan® system allows for computer analysis of the 23 – 24 biochemical tests for onward translation into an 8 digit biotype number based on which the organism is identified from its data base. Results of antibiotic susceptibility tests are in minimum inhibition concentrations (MIC). Results interpretation of breakpoints is based on NCCLS Document M100 – S13 (NCCLS, 2003). The MIC of tests organisms are compared to those of the recommended American type culture collections (ATCC). The MicroScan® panels utilize *Escherichia coli* ATCC 25922 and *Pasteurella aeroginosa* ATCC 27853 as quality control organisms.

Bacteria that were not named by the Walkaway system were identified based on their biochemical results with the aid of online Gideon Informatics (1994 – 2013), as well as with reference to Barrow and Feltham (1993) and Garrity *et al.,* (2005).

2.2. *Plasmid profile and Curing*

Plasmid DNA profile and curing assay for antibiotic resistant bacteria isolates were carried out as described by Birnboim and Doly (1980) with some modifications. To isolate the plasmids, a volume of nutrient broth was inoculated with aliquot collected from overnight bacterial culture grown in nutrient broth containing antibiotics for 24 hours at 37°C. The inoculated nutrient broth was incubated for 18-24 hours to allow growth of the bacteria. Agarose gel was run and plasmid DNA visualised on UV trasilluminator. Molecular weights of plasmids were determined by comparing them with plasmid standards. DNA size standard DNA molecular weight marker II (Roche Applied Science, Germany), was used as positive control

In curing plasmid DNA, a volume of nutrient broth was inoculated with aliquot collected from overnight bacterial culture grown in nutrient broth containing antibiotics for 24 hours at 37°C. The inoculated nutrient broth was incubated for 18-24 hours to allow growth of the bacteria. Sodiumdeodecyl sulphate (SDS) curing agent was added to bring the concentration to 1% (w/v) SDS, followed by incubation for 24 – 48 hours at 37°C ensuring there was growth. Freshly prepared nutrient broth was inoculated with an aliquot of cured culture and incubated for 24 hours at 37°C. Plasmid DNA was isolated from the broth culture as described above. Agarose gel electrophoresis was run and plasmid DNA visualised on UV transilluminator to confirm curing.

3.0 Results

A total of 125 bacterial isolates were obtained from the bacteriological investigations carried out on causes of high mortalities in commercial flocks of poultry studied. Bacteria isolated were *Escherichia coli* (59), *Salmonella* Enteritidis (17), *Salmonella* Pullorum (1), *Salmonella* Gallinarum (24), *Citrobacter youngae* (4), *Klebsiella ozanae* (2), *Klebsiella variicola* (2), *Enterobacter cloaceae* (4), *Hafnia alvei* (1), *Pasteurella gallinarum* (5) *and Pasteurella multocida* (6).

The bacterial isolates showed resistance to 6-23 of the 27 antibiotics tested. Tetracycline and ampicilllin were found as the least potent drugs, with 90 and 90.91% resistance respectively (Table 1). Thirty-four (85%) of the 40 bacterial isolates treated for plasmid profile were found to be carrying plasmids (Figure 1). Plasmid DNA size 11.50 kbp was commonly observed (about 65%) among all the plasmid borne mdr bacteria, in all *E. coli* biotypes (all the *E. coli* screened possess plasmids), *Hafnia alvei*, *Klebsiella varricola* and among *Salmonella* serotypes. Plasmid DNA size 10kbp was observed in *Citrobacter youngae* as well as in some *Salmonella* species (incidence of about 22% in plasmid borne bacteria). As well as a 26.3 kbp plasmid DNA in *Enterobacter cloaceae* and a 6.6 kbp plasmid DNA in *Salmonella* Enteritidis. The susceptibility of the plasmid bearing bacteria to antibiotics was enhanced following plasmid curing with sodium deodecyl sulphate (Table: 2).

4.0 Discussion

The isolated bacteria from clinical poultry cases in this study namely, *Citrobacter youngae*, *Klebsiella varricola, Enterobacter cloacae, Hafnia alvei, Klebsiella* species, *Salmonella* species and *Pasteurella* species are commonly isolated from poultry, but in Nigerian poultry, they have not been frequently reported.

Escherichia coli is a Gram negative bacterium found in the faecal samples of most of the animals. *E. coli* causes a various forms of disease in poultry: broilers, turkeys and egg layers. Diseases caused by *E. coli* are called colibacillosis and may infect all classes and ages of poultry. High mortality in Nigerian poultry associated with multidrug resistant *E. coli* had been reported earlier (Okiki and Ogbimi, 2008, 2010a,b). *E. coli* associated with high mortality in Nigerian poultry was found to show great diversity in biochemical and antibiotic resistant patterns (Okiki and Ogbimi, 2010b).

Citrobacter youngae was first defined by Brenner *et al.* (1993). Miki *et al.* (1996) re-examined the 90 reference strains that had earlier been listed in the scheme for *Citrobacter freundii*. Brenner and his collaborators found that 40 of these strains belong to *C. youngae*, 25 to *C.brakii,* 13 to *C. werkmanii* and only 3 to the redefined *C.*

freundii. C. youngae is found in human stools, urine and wound, isolated from animals and food. Prevalence of drug resistant *Citrobacter* species has recently been reported in goose eggs by Jahangtigh (2013).
Klebsiella species are frequently isolated from poultry. Fielding *et al.* (2012) isolated *Klebsiella ozaenae* and *K. rhinoscleromatis* in 96 and 6 isolates respectively out of 102 chicken samples tested (chicken obtained from a local vendor in South Africa). Both *Klebsiella ozaenae* and *K. rhinoscleromatis* are known to cause clinical chronic rhinitis and are almost exclusively associated with people living in areas of poor hygiene.
Hafnia alvei has been reported to cause septicaemia, respiratory tract infections, meningitis, urinary tract infections, abscesses, and wound infections. In most cases, however, it has been found in mixed culture and seems to be an opportunisty that produces infections in patients with some underlying illness or predisposing factor such as diabetes, chronic renal failure, chronic obstructive disease, malignancy and HIV infection (Garrity *et al.*, 2005). Casagrande *et al.* (2004) reported an outbreak of disease caused by *Hafnia alvei* in pullets. Cloudy swelling and the fatty degeneration of liver associated with splenic lymphocytic depletion were observed as the most preminent lesions.
Enterobacter cloacae is part of the normal intestinal flora, infectious disease due to endogenous *E. cloacae* might be a result of both host predisposing factors and the bacterial virulence determinants. Cox *et al.* (1983) isolated *E. cloaceae* from poulry feed and meat samples.
Salmonella Pullorum and *Salmonella* Gallinarum are highly host adapted to chicken and turkey. Pullorum disease, caused by *Salmonella* Pullorum, usually causes high mortality (up to 100%) in young chicken and turkeys within the 1st 2 to 3 weeks. In adult birds mortality could be high, but in most cases there may be no clinical signs. Pullorum used to be very common, but it has been eliminated in most commercial poultry stocks. There could be however occurrence in most other avian species such as guinea fowls, sparrow and quail. Mammal pullorum outbreak is rare, however experimental infections have been reported in many mammals such as rabbits, guinea pig, kittens, dogs, swine, wild rats and chimpanzee among others. (Kahn *et al*., 2010). Fowl typhoid, caused by *Salmonella* Gallinarum, is a disease of high economic importance in the poultry industry. The disease is egg transmitted, but could be transmitted horizontally by spreading among growing and matured birds. Mortality is usually high in young birds but much higher in older birds (Kahn *et al*., 2010).
Pasteurella multocida often exists as a commensal in the upper respiratory tracts of many livestock, poultry, and domestic pet species, especially cats and dogs. *Pasteurella multocida* causes fowl cholera in birds. Acute fowl cholera is often associated with large number of dead birds without previous signs. In chronic fowl cholera, lesions can localise in sternal bursae, wattles, joints and tendon sheats. *P. multocida* may infect a wide variety of animals, but strains from non-avian host do not cause fowl cholera (Kahn *et al*., 2010).
The high level of resistance to antibacterial agents reported in this study is food for thought. The antibacterial agents commonly used as either growth promoter or for prophylaxis such as tetracycline, ampicillin, chloramphenicol and trimethoprim are worst affected. Gentamycin, norfloxacin and nitrofurantoin, which are drugs of choice in the Nigerian poultry industry when birds are sick, gave 60, 53.48 and 54.56% inefficiency respectively. These resistant patterns could be due to drug misuse and abuse by farmers. Resistance were equally obtained against antibacterial agents that are not commonly used in Nigerian poultry industry, such as the cephalosporins, carbapenems, piperacillin, ticacillin, amikacin, tobramcycin and levofloxacin. This may suggest that such resistance may be genetic, with the presence of genes that provide broad spectrum resistance to many antibacterial agents. It has been reported that clinical bacterial isolates (*Klebsiella oxytica*, *K. pneumoniae* and *E coli*) with increased MICs (➾2µg/ml) of ceftazidine, aztreonam or MICs of ➾2µg/ml or ➾8µg/ml (depending on panel type) of cefpodoxine should be suspected for habouring an extended spectrum beta-lactamase, ESBL (NCCLS, 2003; Dade Behring Inc, 2004). In this study resistance to ceftazidine and cefpodoxine were 52.73 and 49.10% respectively based on MICs >1µg/ml for ceftazidine and >4µg/ml for cefpodoxime (these are the break points for the panel used). The result of the resistance to cefpodoxine obtained in this study suggests the presence of ESBL among the bacterial isolates.
The incidence of 65% of 11.5 kilobase-pair (kbp) plasmid as well as 23% of 10kbp plasmid is an indication of plasmid epidemics. These plasmid sizes, being very close, may likely be the same plasmid. A single plasmid could be observed in several bacterial species in a number of outbreaks and such outbreaks are sometimes called plasmid epidemics (Mayer, 1988). The spread of plasmids carrying a large array of resistance genes among enterobacteriaceae is of great concern because it provides an efficient genetic mechanism of disseminating resistance to multiple antimicrobial drugs (Piorel *et al*., 2007). The pQR1 (Poirel *et al*., 2005) and p1 (Piorel *et al*., 2006) are examples of well characterized plasmids that indicate multiple drug resistance by carrying *bla* $_{VEB-1}$ (ESBL genes) and *qnrA1* (that confer resistance to nalixidic acid and reduced susceptibility to fluroquinolones) together with aminoglycoside resistance genes *aadB, aacA1* and *aadA1*, as well as chloramphenicol resistance gene *cmlA*, rifampin resistance *aarr2*, disinfectant resistance gene *qac1* and sulfonamides resistance gene *su11*.
Apart from low production performances and the overall economical impact of multiple drug resistant bacteria on poultry industry, exposed poultry workers are at risk. This could result in long period of hospitalization and

loss of work hours. Johnson and Hall (1988) had earlier suggested that many of the drug-resistant human isolates were more likely to have originated in poultry or a similar non-human reservoir. Physical ill-health, anxiety and depression associated with occupational exposure of poultry workers to agents in poultry air in Nigeria has been reported by Okiki *et al*. (2013).

Because of ever emerging multi-drug resistant bacteria, there is need to constantly monitor, isolate, type cultures and have organisms prevalent in the country stored in reference laboratories. Antibiotic susceptibility testing should guide treatment in all infections where organism can be cultured in both human and veterinary medicine.

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Table 1: Susceptibility of bacterial isolates from poultry to various antimicrobial agents

Drugs	**Susceptibility (%)**
A. Penicillins:	
* Ampicillin	9.09
* Amoxicillin/K Clavulanate	54.54
* Piperacillin/Tazobactan	59.75
* Tircacillin/K Clavulanate	85.45
B. Cephalosporins	
1st * Cefazolin	23.64
2nd * Cefotetan	75.54
* Cefurozime	43.65
3rd * Cefotaxime	89.28
* Cefpodoxime	50.90
* Ceftazidime	47.27
* Ceftriaxone	90.91
4th * Cefepine	81.8
C. Aminoglycosides	
* Gentamycin	40.00
* Amikacin	92.72
* Tobromycin	61.81
D. Quinolones	
1st * Nalixidic acid	12.00
2nd * Norfloxacin	46.52
* Ciprofloxacin	75.91
3rd *Levofloxacin	80.91
E. Tetracycline	10.45
F. Chloramphenicol	16.45
G. Trimethoprim/sulphamethazaxole	18.18
H. Nitrofurantoin	45.44
I. Carbapem:	
* Imipenem	77.54
*Meropenem	94.18
1st, 2nd, 3rd & 4th = Generations of drugs	

Table 2: Enhancement of antibiotics effectiveness on some bacterial isolates following plasmid curing

BACTERIA	RESISTANT DRUGS BEFORE CURING	RESISTANT DRUGS AFTER CURING
Salmonella pullorum 4006706-2	AG.N.C.T.GN.CIP.AM.NA.CF	AG.NA
Salmonella pullorum 4006706-2	N.C.T.GN.CIP.AM.NA.	T.NA.AM
Escherichia coli 7711700-2	N.GN.CIP.AM	NIL
Escherichia coli 7711700-2	NB.T.GN.CIP.AM	NIL
Escherichia coli 7715700-2	N.C.T.GN.CIP.AM.NA.CF	GN.NA
Escherichia coli 7715700-2	C.T.GN.AM.NA.CF	NIL
Salmonella enteritidis 7356724-0	GN.CIP.AM.NA.CF	NIL
Salmonella enteritidis 7356724-0	AG.N.C.T.C.AM.NA.CF	NIL
Salmonella enteritidis 5356704-2	N.T.NA	NIL
Enterobacter cloaceae 7514337-2	AG.N.C.T.GN.CIP.AM.NA.CF	T.CF
Enterobacter cloaceae 7514337-2	AG.C.T.GN.CIP.AM.NA.CF	AM.NA
Klebsiella variicola 4002015-0	NB.T.GN.CIP.NA	NIL
Hafnia alvei 5304727-6	AG.N.C.T.GN.CIP.AM.NA.CF	T.GN.AM.

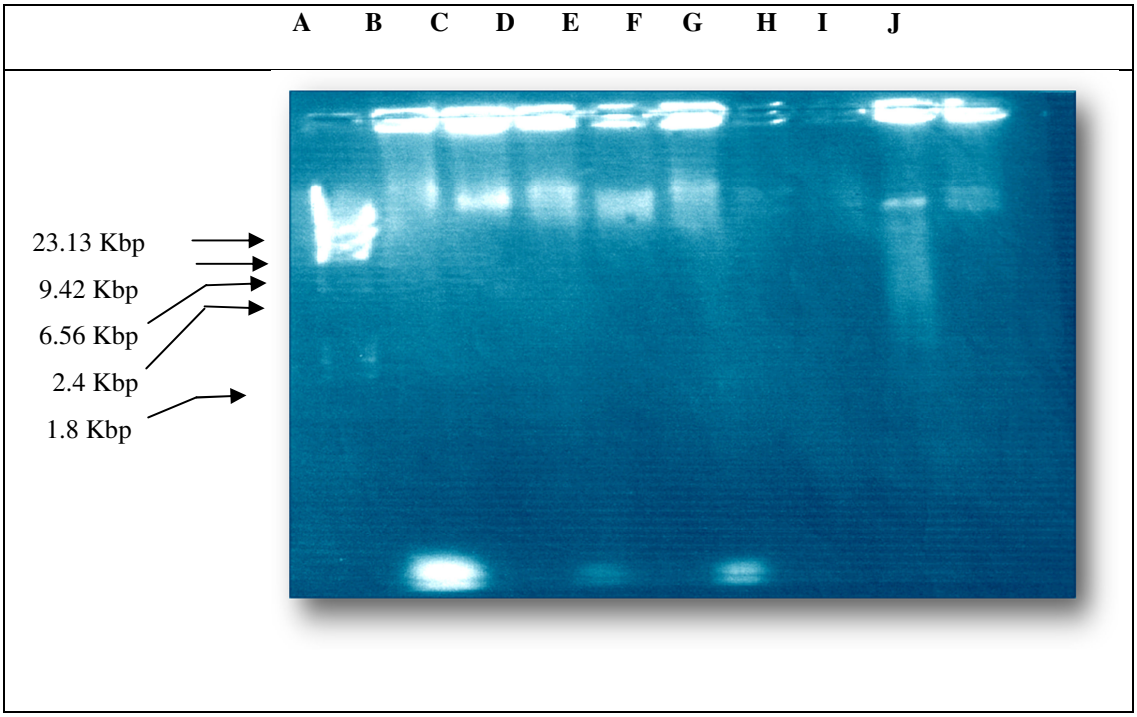

Figure 1: Agarose electrophoresis of plasmid DNA of multidrug-resistant bacteria isolates from poultry. Well A: Standard DNA Molecular Weight Marker II

Wells B – J: Isolated plasmids from multidrug resistant bacteria