PLASMID-BORNE ANTIMICROBIAL RESISTANT BACTERIA ISOLATED FROM POULTRY LITTER: IMPLICATION FOR CROP PRODUCTION

BY

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Abstract

Antimicrobial resistance in poultry manure and implication for crop quality was investigated in this study. Poultry litter samples obtained from Delta State University farms located in Abraka (farm A) and Anwai (farm B) campuses were used in this study. Bacterial isolates were identified by cultural, morphological and biochemical characteristics following standard methods. The distribution of bacteria from poultry litter was Escherichia coli (26.17%), which was the most predominant, followed by Staphylococcus aureus (11.41%), Streptococcus pyogenes (10.74%), Aeromonas hydrophilia (10.07%), klebsiella pneumonia (8.72%), Enterobacter aerogenes (8.05%), Proteus mirabilis (6.71%) Listeria monocytogenes (6.04%), Bacillus cereus (5.37%), Pseudomonas aeroginosa (3.36%) and Salmonela enterica (3.36%). Antimicrobial susceptibility test was carried out on all isolates against 19 different antibiotics using disc diffusion methods. In order to determine the role plasmids play in resistance, curing tests were performed using Sodium dodecyl sulfate as the curing agent. The total bacteria count ranged from 5.0 x 10^4 to 9.9 x 10^4 in farm A and 1.5 x 10^4 to 8.0 x 10^4 in farm B. All isolates were multi drug resistance. No isolate (0.00%) were found to be resistant to amoxyxillin-clavulanic acid, ciprofloxacin and ofloxacin. Result also indicated that all isolates haboured resistance to one or more antibiotics on the plasmid. Poultry litter was found to contain a diversity of pathogens that haboured antibiotic resistance on plasmids. Raw fruits and vegetables are an essential part of people's diet. These fruits and vegetables are usually grown with poultry litter which may be contaminated. Considering the roles plasmid play in dissemination of resistance, it is important that poultry litter be adequately treated by composting or by anaerobic digesters before use.

Key words: Plasmid borne resistance, antibiotic resistance, composting, poultry litter.

Introduction

Poultry litter, has always been considered to be one of the most valuable animal wastes as organic fertilizer due to its high nutrient content. Essentially, poultry manure supply most essential plant nutrients and serve as a soil amendment by adding organic matter which help improve the soil's moisture and nutrient retention. It is readily available locally and can reduce fertilizer cost in crop production. Currently, the United States Department of Agriculture (USDA) allows poultry manures to be used as fertilizer to grow fruits, vegetable and grains meant for human consumption. There are no restrictions on the use of poultry manure for crop production. However, a number of human pathogens such as *Escherichia coli*, *Salmonella enterica, Campylobacter jejuni, Clostridium perfrigens, Listeria monocytogene* are associated with poultry manure and have been implicated in foodborne outbreaks (Chinivasagam, Redding, Runge and Blackall, 2010; CDC, 20 11; Wilkinson, Tee, Tonkins, Hepworth and Premier, 2011). Manures not only provide a favourable environment for pathogens to survive but also for re-growth due to availability of nutrient as well as protection from ultra violet (UV) radiation and extreme temperature (Rogers and Haines, 2005).

Contamination of fresh produce with fecal pathogenic bacteria in the agricultural environment has been documented as the main cause of numerous food poisoning outbreaks (Doyle, 2008). Antibiotics are valuable to cure or prevent respiratory disease and infections in poultry. This has resulted in antimicrobial resistance. Antibiotics are routinely added to animal feed in sub-therapeutic doses for growth promotion of animals produced for human consumption. This practice may lead to a selection of resistant microbial population (including pathogens) in the native micro-biota of the animal and the local environment due to resistance to antibiotics. Resistance genes are often found on plasmids, which are extrachromosomal DNA molecules that can exist independent of the chromosome. (Prescott, Hardy and Klein 2001). Plasmids are commonly found in bacteria. Resistance genes encoded on plasmids are often located within genetic elements such as transposoons or integrons (Lu, Sanchez, Hofacre, Maurer, Harmon, and Lee 2003). A resistance gene that has emerged on a plasmid located within a transposon or an integron may be transferred to other strain and species (O'Brien, 2002)

Plasmid-mediated gene transfer plays an important role in the rapid dissemination of the resistance gene. There is therefore the need to inactivate organisms harbouring plamids before manure is applied on crops as fertilizer. This study was therefore carried out to; 1) evaluate the total bacterial load and the distribution of bacterial isolates from poultry litter; 2) determine the antimicrobial resistance patterns of the bacterial isolates from poultry liter; 3) determine the role of plasmids in resistance by curing; and 4) point out the human health and crop implications.

Materials and Methods

Poultry litter samples were collected aseptically into sterile plastic bags from layers of litter in Delta State University farms at Abraka campus (farm A) and Anwai campus (farm B) of the university. Samples were collected both from the surface and core of the layers of poultry litter. Samples were placed in ice packs and transported within 2 hours of collection to Microbiology laboratory, Delta State University, Abraka.

Each sample was mixed vigorously by shaking for 1 minute. Five grams of litter was then transferred to 45ml of 0.1% peptone water and vortexed for 1 minute

(Islam, Jennie, Doyle, Sharad, Miller, and Jiang 2004). Serial dilution was carried out using 0.1% peptone water as diluents. An aliquot (100µl) of the different dilutions were pour plated on nutrient agar, MacConkey agar, blood agar and Salmonella-Shigella agar. Plating was done in duplicate. Plates were incubated at 37^{o}C for 24hrs. After incubation, colonies that developed on the plates were counted to obtain total bacteria count.

Isolates were sub-cultured onto fresh agar plates to obtain pure cultures. Identification of pure isolates was based on cultural, morphological and biochemical characteristics using standard methods (Cheesbrough, 2000).

Antimicrobial resistance test was performed by the agar disc diffusion method following National Committee for Clinical Laboratory Standards (NCCLS, 2001). A suspension of the organism matching 0.5 Macfaland turbidity standard were inoculated on the surface of Mueller Hinton agar (Oxoid, England) and allowed to dry. Multi disc containing the following antibiotics; Ceftazidime (Caz - 30µg), Cefuroxime (Crx - 30µg), Gentamicine (Gen - 10µg)), Ciprofloxacin (Cpr - 5µg)), Ofloxacin (Ofl - 5µg)), Amoxycillin-Clavulani acid (Aug - 30µg), Nitrofuratoin (Nit - 300µg), Ampicillin (Amp - 10µg), Cefixime (Cxm - 5µg), Meropenem (10µg) Ceftiaxone (Ctr - 30µg), Erythromycin (Ery- 5µg), Cloxacillin(Cxc - 5µg) and Trimethprim-sulphameyhoxazole - 25µg) were used. The disc were placed on the surface of inoculated plates. The plates were incubated at 37^{o}C and observed for zone of inhibition after 24hrs.

Plasmid curing was carried out by the modification of Olukoya and Oni, 1990 using sodium dodecyl sulphate (SDS). The isolates were treated with 10% SDS. The colonies were later subcultured onto Mueller Hinton agar (Oxoid, England). To verify plasmid loss, the cells were tested for antibiotic resistance as previously described.

Result and Discussion

Poultry litter contains a large and diverse population of microorganisms. Inspite of this, poultry litter are applied to soil as source of nutrients to crops (Lu *et al*., 2003; Ngodigha and Owen, 2009; Bolan, Szog, Chuasavathi, ., Seshadri., Rothrock, and Panneerselvam, 2010). The total bacteria count in farm A ranged from 5.0 X 10^4 to 9.9 X 10^4 and 1.5 X 10^4 to 8.0 X 10^4 (farm B) (Table 1).

Table 1: Total bacterial count of poultry litter (number of colonies X 10^4 cfu/g)

Location	TBC (cfu/g)
Farm A	9.0
Farm A	9.9
Farm A	8.0
Farm A	9.7

Farm A	5.0
Farm A	8.8
Farm B	2.8
Farm B	8.0
Farm B	6.7
Farm B	4.2
Farm B	1.5
Farm B	5.9

One hundred and forty-nine bacteria were isolated from the poultry litter samples. The degree to which manure related pathogens may be involved in diseases outbreaks is poorly investigated due to difficulties in identifying etiologic agents and sources of contamination and because many cases of illness go unreported. Hazard Analysis Critical Control Point (HACCP), a system which identifies, evaluates and controls hazards are yet to be put in place. As such information on source of purchasing of vegetables, transportation, storage and type of manure used in fertilizing vegetable are often not available. The paucity of information makes it difficult to pin point the sources of contamination.

Salad (a mixture of raw vegetables and/or fruits) (Arvanitoyannis, Bouletis, Papa, Gkagtzia, Hadjichritodoulou, and Paploucas, (2011) or African salad (a special salad recipe native to Nigeria that contains raw vegetable) (Oranusi *Braide*, Eze, and Chinakwe, 2013) are ready to eat foods sold in the streets and towns of many developing countries. These foods are patronized by many consumers because fruits and vegetables are well known sources of useful nutrients. These vegetables used in preparing these salad delicacies are often fertilized with poultry litter or other organic fertilizers that are inadequately composted and they may act as a source of reservoir for many microorganisms (Benchat, 2002; Taban and Halkman, 2011). Researches on microbiological quality of fruits and vegetables have revealed heavy loads of microbial contaminants belonging to either *Pseudomonas* group or Enterobacteriaceae (Lund, 1992; Uzeh, Alade, and Bankole,., 2009 Osamwonyi, Obayagbona, Aborishade, Olisaka, Uwadiae, and Igiehon, 2013;). Consumption of such fruits and vegetables present microbiological risk.

Wogu and Iwezeua (2013) isolated *Slamonella*, *E. coli* and *Staphylococcus aureus* in their study on ready to eat salad. They suggested that these pathogens may be from contaminated vegetables planted with animal droppings. The distribution of the isolates according to different genera is shown in Table 2.

Table 2: Distribution of bacterial isolates from poultry litter

Isolates	No. of Isolates (%)
Escherichia coli	39(26.17)
Aeromonas hydrophilia	15(10.07)
Klebsiella pneumonia	13(8.72)
Enterobacter aerogenes	12(8.05)
Proteus mirabilis	10(6.71)
Pseudomonas aeruginosa	5(3.36)
Salmonella enterica	5(3.36)
Staphylococcus aureus	17(11.41)
Streptococcus pyogenes	16(10.74)
Listeria monocytogenes	9(6.04)
Bacillus cereus	8(5.37)
Total	149

Some of these organisms such as *Streptococcus* are normal inhabitant of the intestine and therefore, not a health risk to humans. Others such as *Escherichia coli, Salmonella enterica* and *Listeria monocytogens* have been implicated in foodborne illness (Islam, Das, Hossain, Lucky, and Mostafa 2003; CDC, 2011). They present epidemiological problems in poultry breeding and are of public health importance (Heuer and Smalla, 2007). An active surveillance data on foodborne diseases from the United States reveal that among the pathogens associated with foodborne outbreaks, *Salmonella, E. coli, Campylobacter* and *L. monocytogens* are responsible for the majority of outbreaks (CDC, 2013). These pathogens can be transmitted to humans directly through contact with poultry litter or indirectly through contaminated food crops.

Normal intestinal flora are not health risk in humans but they can develop resistance. Large quantities of antimicrobials are used to treat, prevent disease and to promote animal growth (Prescott, 2001). These antimicrobials are added to feed or drinking water at sub therapeutic levels for extended periods of time (weeks or months). Such a misuse and /or unsuitable usage result in normal commensal intestinal flora developing resistance to antimicrobials used. Similarly, this misuse also increases the possibility of selecting pathogenic organisms resistant to antibiotics. Poultry manure therefore has become the single largest reservoir of antimicrobial resistance arising from animal production (Chee-Sanford *et al*., 2008).

Antimicrobial susceptibility test was carried out on the isolates using disk diffusion method. One hundred percent resistance (100%) was observed in most of the isolates (*E. coli*) to ceftazidime, cefuroxime, cefixime, ampicillin and meropenem. Most isolates were 0.0% resistance to the quinolones (ciprofloxacin and ofloxacin), amoxicillin-clavulanic acid and nitrofuratoin. Low levels of resistance has also been reported by Grahama, Evans, Price and Silbergeld (2009) to the quinolones. Consistent with other researches, low resistance to gentamicin have been reported. This is attributed to its low level of usage and absorption by poultry (Salehi and Bonab, 2006; Ginns *et al*., 1996).

Table 3: Antimicrobial resistance pattern of gram negative isolates from poultry

Isolates (number)	Number of isolates resistant to antibiotics (%)										
	CAZ	CRX	MEM	CXM	AMP	AUG	NIT	GEN	CPR	OFL	SXT
Escherichia coli (39)	39 (100.00)	39 (100.00)	32 (82.05)	39 (100.00)	39 (100.00)	0 (0.00)	4 (10.26)	19 (48.72)	0 (0.00)	0 (0.00)	32 (82.51)
Salmonella enterica (5)	5 (100.00)	5 (100.00)	5 (100.00)	5 (100.00)	5 (100.00)	0 (0.00)	0 (0.00)	3 (60.00)	0 (0.00)	0 (0.00)	5 (100.00)
Pseudomonas aeruginosa(5)	5 (100.00)	5 (100.00)	5 (100.00)	5 (100.00)	5 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	5 (100.00)
Klebsiella pneumonia.(12)	12 (100.00)	12 (100.00)	12 (100.00)	12 (100.00)	12 (100.00)	0 (0.00)	0 (0.00)	5 (41.67)	0 (0.00)	0 (0.00)	12 (100.00)
Enterobacter aerogenes.(13)	13 (100.00)	13 (100.00)	13 (100.00)	13 (100.00)	13 (100.00)	0 (0.00)	7 (53.85)	9 (62.23)	0 (0.00)	0 (0.00)	13 (100.00)
Proteus mirabilis.(10)	10 (100.00)	10 (100.00)	10 (100.00)	10 (100.00)	10 (100.00)	0 (0.00)	5 (50.00)	0 (0.00)	0 (0.00)	0 (0.00)	5 (50.00)
Aeromonas hydrophilia.(15)	15 (100.00)	15 (100.00)	15 (100.00)	11 (73.33)	15 (100.00)	0 (0.00)	7 (46.67)	3 (20.00)	0 (0.00)	0 (0.00)	3 (20.00)

CAZ - Ceftazidime, CRX - Cefuroxime, MEM – Meropenem,CXM – Cefixime, AMP – Ampicillin, AUG - Amoxycillin-Clavulanic acid, NIT – Nitrofuratoin, GEN – Gentamicine, CPR – Ciprofloxacin, OFL – Ofloxacin and SXT - Trimethprim-sulphameyhoxazole

Table 4: Antimicrobial resistance pattern of gram positive isolates from poultry litter

Isolates (No.)	Number of isolates resistant to antibiotics (%)							
	CAZ	CRX	CXC	CTR	GEN	ERY	OFL	AUG
Staphylococcus aureus (17)	4 (23.53)	17 (100.00)	17 (100.00)	12 (70.59)	8 (47.06)	0 (0.00)	0 (0.00)	12 (70.59)
Streptococcus pyogenes(16)	4 (25.00)	13 (81.25)	16 (100.00)	3 (18.75)	9 (56.25)	8 (50.00)	0 (0.00)	16 (100.00)
Listeria monocytogenes. (9)	9 (100.00)	9 (100.00)	9 (100.00)	5 (55.56)	9 (100.00)	8 (88.89)	0 (0.00)	4 (44.44)
	8	8	8	8	8	7	0	7

Bacillus cereus(8)	(100.00)	(100.00)	(100.00)	(100.00)	(100.00)	(87.50)	(0.00)	(87.50)

CAZ - Ceftazidime, CRX - Cefuroxime, CXC - Cloxacillin CTR - Ceftiaxone, GEN – Gentamicine, ERY- Erythromycin, OFL – Ofloxacin and AUG - Amoxycillin-Clavulanic acid. gAmong the antibiotics used for sensitivity test, aminoglycosides (gentamicin), macrolides (erythromycin) and fluoroquinolone (ciprofloxacin and ofloxacin – through were used until banned in 2005) are used in poultry production. Though penicillin and ceftiofur which are also used in poultry production was not used in this study but penicillin is a β-lactam antibiotics and ceftiofur is a third generation cephalosporin. Some β-lactam antibiotics used for sensitivity test in this study were the cephalosporins such as ceftazidime, cefuroxime, cefixime. Resistance to these related antibiotics can be mediated by similar mechanisms (Ozgumus, Sandalli, Sevim, Celik-sevim, and Sivri, 2009). Resistance bacteria can pass their resistance genes to other bacteria (Chang, 2014). Some of these genes can confer resistance to other antibiotics that were not used on the animal (Dutil, 2010). It is not surprising therefore that all the isolates in this study were multi drug resistance (resistant to 3 or more antibiotics). Multiple drug resistance stems from clustering of multiple antimicrobial resistance genes together primarily on mobile genetic elements. This clustering of genes can affect the persistence of antibiotic resistance because eliminating only one or two antibiotics may not reduce the prevalence of the cluster. Such reservoir of resistant bacteria originating from the use of poultry litter as manure eventually may contaminate fruits and food vegetables and be picked up by other animals or humans transmitting the resistance (likely by plasmids) genes further.

Table 5: Antimicrobial resistance profile of gram negative bacteria after curing for 48hr

Isolates (number)	Number of isolates resistant to antibiotics (%)										
	CAZ	CRX	MEM	CXM	AMP	AUG	NIT	GEN	CPR	OFL	SXT
Escherichia coli (39)	38 (97.44)	38 (97.44)	29 (74.36)	39 (100.00)	4 (10.26)	0 (0.00)	0 (0.00)	18 (46.15)	0 (0.00)	0 (0.00)	29 (71.36)
Salmonellaenterica (5)	5 (100.00)	5 (100.00)	5 (100.00)	0 (0.00)	5 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	5 (100.00)
Pseudomonas aeruginosa(5)	5 (100.00)	5 (100.00)	5 (100.00)	5 (100.00)	5 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	5 (100.00)
Klebsiella pneumonia.(12)	12 (100.00)	12 (100.00)	10 (83.33)	9 (75.00)	12 (100.00)	0 (0.00)	0 (0.00)	4 (33.33)	0 (0.00)	0 (0.00)	12 (100.00)
Enterobacter aerogenes.(13)	13 (100.00)	13 (100.00)	5 (38.46)	3 (23.08)	13 (100.00)	0 (0.00)	7 (53.85)	3 (23.08)	0 (0.00)	0 (0.00)	13 (100.00)
Proteus mirabilis.(10)	10 (100.00)	10 (100.00)	5 (50.00)	0 (0.00)	10 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	5 (50.00)
	15	15	12	4	15	0	0	0	0	0	3

Aeromonas hydrophilia.(15)	(100.00)	(100.00)	(80.00)	(26.67)	(100.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(20.00)

CAZ - Ceftazidime, CRX - Cefuroxime, MEM – Meropenem,CXM – Cefixime, AMP – Ampicillin, AUG - Amoxycillin-Clavulanic acid, NIT – Nitrofuratoin, GEN – Gentamicine, CPR – Ciprofloxacin, OFL – Ofloxacin and SXT - Trimethprim-sulphameyhoxazole

Table 6: Antimicrobial resistance profile of Gram positive bacteria after curing for 48hr

Isolates (No.)	Number of isolates resistant to antibiotics (%)							
	CAZ	CRX	GEN	CTR	ERY	CXC	OFL	AUG
Staphylococcus aureus (17)	4 (23.53)	8 (47.06)	0 (0.00)	4 (25.53)	0 (0.00)	17 (100.00)	0 (0.00)	8 (47.06)
Streptococcus pyogenes(16)	4 (25.00)	4 (25.00)	(0.00)	0 (0.00)	4 (25.00)	16 (100.00)	0 (0.00)	4 (25.00)
Listeria monocytogenes. (9)	4 (44.44)	4 (44.44)	4 (44.44)	4 (44.44)	0 (0.00)	8 (88.89)	0 (0.00)	0 (0.00)
Bacillus cereus(8)	7 (87.50)	4 (50.00)	4 (50.00)	4 (50.00)	4 (50.00)	8 (100.00)	0 (0.00)	6 (75.00)

CAZ - Ceftazidime, CRX - Cefuroxime, CXC - Cloxacillin CTR - Ceftiaxone, GEN – Gentamicine, ERY- Erythromycin, OFL – Ofloxacin and AUG - Amoxycillin-Clavulanic acid.

In order to determine the location of resistance, plasmid curing was carried out using sodium dodocyl sulfate (SDS) as the curing agent. Loss of antibiotic resistance was associated with plasmid loss which implies plasmid borne resistance. From the result, it appears that all the isolates haboured resistance to one or more antibiotic on the plasmid. The resistance of bacteria and plasmid has been reported recently to be transmitted to humans from animals (Mahmood and Bal Bujukunal, 2014). Leverstein-van Hall *et al.*, 2011 reports a range of *E. coli* isolates from human and poultry harbouring the same plasmids. Mades *et al.*, 2012 demonstrated that $Incf_{11}$ plasmid circulated between diverse clones of *E. coli* from humans and animals.

The quality of manure increases when composted. As a result of composting they become more stable and nutrients are releasesd more slowly than they are from raw manure (Zhao, Knowlton and Love, 2008; USDA, 2009). Recent research also suggests that composting may promote antimicrobial degradation (Remaswamy, Prasher, Patel, Hussain, and Barrington, 2010). Anaerobic digesters can also be used to mitigate the possibilities of crops becoming contaminated. The benefit of using anaerobic digesters include reduction in pathogens, reduces greenhouse emission (methane and carbon dioxide) and minimization of odors (USDA, 2011). The implication therefore is that crop farmers stand to benefit tremendously with the use of composted and anaerobic digested manure.

Conclusion

Poultry litter was found to contain a diversity of pathogens that harboured antibiotic resistance on plasmids. Plasmids play important role in dissemination of resistance. The possibility of humans consuming poultry litter contaminated food crops especially raw vegetables may be high (though food poisoning outbreaks are rarely reported) because in recent times most food crops are fertilized using contaminated poultry litter. In order to reduce food borne diseases, it is important that poultry litter be adequately treated by composting and by anaerobic digestion before use. In addition manure should be incorporated into the soil and polytene mulch can be used to cover the soil. Consumers of freshly harvested vegetables, especially from farms where poultry litter was applied should ensure proper handling and washing of the vegetables before consumption.

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