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The assessment of contamination level of multidrug resistant bacteria and antibiotic residue in chicken manure

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This study aimed to assess the contamination level of chicken litter by antibiotic residues and multidrug resistant (MDR) bacteria. A total of 28 chicken litter samples were aseptically collected in Abidjan poultry farms in December 2021. The bacteria including *Salmonella, Escherichia coli* and *Staphyloccocus aureus* were isolated on specific culture media and were identified using biochemical tests. Antibiotics susceptibility test of the isolates was performed by the disk diffusion method. Finally, the antibiotic residues were detected in litter samples by HPLC. Among the analyzed samples, 21 were positive for the three bacteria. Moreover, *S. aureus* isolates were resistant to tetracycline, clindamycine, trimethoprime, and erythromycine while *E. coli* and *Salmonella* strains were resistant to minocycline, nalidixic acid, and tetracycline with resistance rate above 75% for all strains. The MDR including 3 to 6 antibiotic classes were found in 90 and 23.07% of *S. aureus* and *E. coli* strains, respectively. Moreover, chicken litter samples were contaminated by antibiotic residues mainly ciprofloxacine, erythromycine, spiramycine and oxytétracycline with amounts between 0.05 ± 0.002 and 8.41 ± 2.03 mg/kg. These results showed the need to treat chicken litter before being used as fertilizer to reduce their negative impact on environment and health.

Key words: Chicken litter, multidrug resistant bacteria, antibiotic residues, soil, crops pollution.

INTRODUCTION

In Côte d'Ivoire, livestock production and agricultural subsistence farming practices have intensified in recent decades. The poultry sector specifically, has expanded since 2006 when restrictions on import of frozen chicken were introduced (ANADER, 2018). Thus, the annual production had risen from 20,000 tons/year in 2011 to 174,000 tons/year in 2020 (IPRAVI, 2021). With this expansion of the poultry industry in the country, production of poultry litter as a waste product has also increased, further encouraging its use as manure for agriculture system.

In general, this manure is used as fertilizer by many farmers but without prior treatment. In the region of Abidjan in particular, poultry litter is mostly used by

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market gardeners. Indeed, even if farmers have easy access to groundwater for crop irrigation (through spraying water method), they are limited by the low fertility of the coastal sandy soils of Abidjan area. Thus, to satisfy growing urban demand and to improve crop productivity, they have adopted intensive practices, such as application of high rates of poultry manure as fertilizers, which is available locally.

However, besides its rich organic content, poultry litter and manure can be contaminated with various types of pathogens including viruses, bacteria, parasites, and fungi. Indeed, foodborne bacteria such as *Escherichia coli, Salmonella, Staphyloccocus aureus* and *Campylobacter* species have been isolated in poultry intestinal tract and in litter (Viegas et al., 2012).

These bacteria pose a risk of transmission to humans, animals and the environment; especially considering their ability to survive for months in water, soil and crops (Merchant et al., 2012; Oliveira et al., 2012). Moreover, in developing countries as Côte d'Ivoire, there is no surveillance system for antibiotics use during breeding. Thus, besides the risk of microbial contamination, there is an additional concern of transmission of multidrug resistant (MDR) bacteria, due to this reported high use of these antibiotics either as growth promoters or for prophylactic purposes (Dahshan et al., 2015; Goualié et al., 2020; Roth et al., 2018). However, so far, little is known of the microbiological and chemical components of poultry litter used as fertilizer in Côte d'Ivoire.

This study therefore aim to assess the level of contamination in chicken litter use as manure in vegetable culture in Abidjan of antibiotic residues and MDR bacteria in view to evaluate the risks associated to the use of this litter without prior treatment.

MATERIALS AND METHODS

Chicken litter samples collection

A total of 28 litters samples were randomly collected from 16 farms in five municipalities including Yopougon, Songon, Bingerville, and Anyama because of their high poultry production capacity in the District of Abidjan, Côte d’Ivoire. In each farm, the litter was aseptically samplied directely from previously stored bags. Each collected sample was packed in sterile plastic bags and transported to the Biotechnology laboratory of the Félix Houphouët-Boigny University. Among these 28 litters samples, 6 were from Yopougon, 5 were collected in Songon, 9 were from Bingerville and 8 were from Anyama.

Microbiological analyses of the chicken litter

For each sample, 10 g of poultry litter was transferred in 90 ml of Buffered Peptone Water (BPW) (Bio-Rad Marne-la-Coquette, France). The suspension was homogenized before a serial dilution in salt buffer. Then, the enumeration of *E. coli* and *S. aureus* was performed by culture, respectively, on TBX agar (Conda, Madrid, Spain) as described in ISO/TS 16649-3 guidelines and according to the directives of AFNOR V 08- 057-1: 2004 onto Baird-Parker (BP) agar medium (CM 275, Oxoid, UK) supplemented with egg yolk tellurite emulsion (SR 54, Oxoid, UK).

Thus, for each dilution (10^{-1} to 10^{-5}), 0.1 ml was spread onto the specific medium and all plates were incubated at 37°C during 24 h.

Concerning *Salmonella*, the method described by ISO 6579 was used. Thus, the BPW was directly incubated during 18 h at 37°C as pre-enrichment medium. From each pre-enriched sample, 0.1 ml was used to inoculate 10 ml of Rappaport-Vassiliadis broth (Bio-Rad, Marne-la-Coquette, France). Samples were incubated during 19 h at 42°C and *Salmonella* isolation was achieved on Hektoen agar (Bio-Rad, Marne-la-Coquette, France) medium at 37°C for 24 h. After incubation, one typical colony was selected on each medium for morphological (Gram staining) and biochemical identification by using API®20E gallery (BIOMERIEUX, France) for *E. coli* and *Salmonella* strains and API® Staph gallery for *S. aureus* isolates. The isolates then were stored at -20°C in liquid medium supplemented with glycerol (25%).

Antibiotics sensitivity test of *E. coli*, *Salmonella* and *S. aureus* isolated from chicken litter

Antibiotic susceptibility tests were carried out on 31 *E. coli*, *S. aureus* and *Salmonella* strains by using the Kirby-Bauer (Bauer et al., 1966) diffusion method in the Mueller-Hinton agar medium. The reference strain notably *E. coli* ATCC 25922 was used as a quality control for the susceptibility testing. The following antibiotics were tested for *E. coli* and *Salmonella*: ampicillin (10 µg), cefalotine (30 µg), cefepime (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), imipenem (10 µg), tetracycline (30 µg), minocycline (30 µg), gentamicine (10 µg), amikacine (30 µg), nalidixic acid (30 µg), norfloxacine (5 µg), ciprofloxacine (5 µg), chloramphenicol (30 µg), and trimethoprime (25 µg). For *S. aureus*, penicilline (1 IU), tobramycine (10 µg), gentamicine (10 µg), minocycline (30 µg), tetracycline (30 µg), erythromycine (15 µg), clindamycine (2 µg), trimethoprime (25 µg), fusidic acid (10 µg), vancomycine (30 µg), rifampicine (5 µg), norfloxacine (10 µg), fosfomycine (200 µg), kanamycine (30 µg), and cefoxitine (30 µg) were tested. All commercial antibiotic dics were purchased from BioRad (France). The inoculated plates were incubated during 24 h at 37°C and the inhibition zones were measured and analyzed according to the CASFM/EUCAST (2022) guidelines.

Antibiotics residues detection in chicken litter

Before the determination of the antibiotic residues in the chicken litter, the samples collected in each commune were mixed to make four large samples. From each large sample, 10 g was collected three times and analyzed separately. Thus, twelve (12) representative antibiotics which were widely used in poultry production system (Goualie et al., 2020) were targeted. Each drug was extracted by ultrasonic method for the classes of macrolides and betalactamin or by using a chemical solvent for sulfonamides, tetracyclines, and fluoroquinolones. Then, the target antibiotics were subsequently analyzed using a high performance liquid chromatography (HPLC). The HPLC Equipment (Waters, USA) consists of the UV visible spectrophotometer (Packard), the systems using a Waters 986 Tunable absorbance detector, a Waters 600 controller, an X-Act degasser, a Waters 717 plusautosampler, a Genesis C18 column (4.6 × 150 mm, 4 µm), a C18 guard column (10 × 4 mm, 4 µm) and a column support (10 mm). For each antibiotic, the standard consisted of a pure solution of the target antibiotic (Sigma-Aldrich). Each test was repeated three times and results were expressed as mean ± standard deviation.

Table 1. Antibiotic resistance profile of *S. aureus* strains.

Antibiotics used	Resistance % (number of strains)	Number of antibiotic classes
K	20 (2/10)	1
E	90 (9/10)	1
CIP	10 (1/10)	1
CMN	80 (8/10)	1
TMP	90 (9/10)	1
VA	30 (3/10)	1
CHL	10 (1/10)	1
TET	90 (9/10)	1
TMP/VA	30 (3/10)	2
TE/CHL/VA/E	10 (1/10)	4
TMP/ TET /CMN/E	30 (3/10)	4
TMP/ TET /CMN/E/K	10 (1/10)	5
TMP/ TET/CMN/E/VA	20 (2/10)	5
TMP/ TET /CMN/E/CIP	10 (1/10)	5
TMP/ TET /CMN/E/VA/K	10 (1/10)	6

K: Kanamycine; E: erythromycine; CIP: ciprofloxacine; CMN: clindamycine; TMP: trimethoprime; VA: vancomycine; CHL: chloramphenicol; TET: tetracycline.

RESULTS

Prevalence and antibiotic profile of the bacteria isolated from chicken litter

A total of 28 poultry litter samples were collected and analyzed for *E. coli, S. aureus*, and *Salmonella* isolation. All tested samples (100%) were positive for *E. coli* and *S. aureus,* while 21 (75%) samples were positive for *Salmonella*. Among these samples positive for *Salmonella* genus, 4 were from Yopougon, 5 were from Songon, 8 were from Bingerville, and 4 were from Anyama.

The bacterial load for *E. coli* was between 2.60×10^2 and $3,8 \times 10^2$ CFU/g, while it was 5.2×10^3 and 9.84×10^4 CFU/g for *S. aureus*. Among the 77 bacteria strains (one confirmed strain per each positive sample), 31 strains including 8 *Salmonella*, 13 *E. coli* and 10 *S. aureus* were selected for the antibiotics susceptibility testing. The frequency of antibiotic resistance for tested strains are shown in Tables 1 to 3. Results show that *S. aureus* strains were found with high resistance to tetracycline clindamycine, erythromycine and trimethoprime with rate above 80% (Table 1). Comparatively to these drugs, low resistance was observed with kanamycine (20%), ciprofloxacine (10%), vancomycine (30%), and chloramphenicol (10%). While, no resistance was observed for gentamycine, tobramycine, fusidic acid and cefoxitine. However, cross resistance to sulfonamides (trimethoprime) and glycopeptides (vancomycine) was observed in 30% of *S. aureus* tested strains. In addition, the Multi-Drug Resistance (MDR) including four, five and six antibiotics families was detected in 9 (90%) of *S. aureus* strains (Table 1).

Among the *E. coli*, 10 (76.92%) were resistant to tetracycline and minocycline belonging to tetracyclines classes and to nalidixic acid (fluoroquinolones) (Table 2). While, low resistance was found against ampiciline (7.69%), gentamycine (15.38%) and to ciprofloxacine (7.69%). Furthermore, the MDR including three antibiotics classes was detected in 3 (23.07%) *E. coli* isolates (Table 2). Among the 15 drugs tested in this work, 6 were efficient against *E. coli* with sensitive rate at 100%.

Concerning the 8 *Salmonella* strains, seven were resistant to one or more drugs tested in this study. The highest percentages of antimicrobial resistance were found for nalidixic acid (87.5%), tetracycline (87.5%) and minocycline (75%). Furthermore, cross-resistance including tetracyclines (minocycline and tetracycline) and fluoroquinolones (nalidixic acid and ciprofloxacine) was observed respectively in 75 and 12.76% of *Salmonella* tested isolates (Table 3). Another cross-resistance case including four or three drugs belonging to tetracyclines and fluoroquinolones was found in *Salmonella* isolates (Table 3). No MDR case was observed in *Salmonella* tested strains.

Antibiotic residues detected in chicken litter

Analysis of chicken litter showed that among the 12 targeted molecules, 7 including ciprofloxacine (fluoroquinolones), erythromycine and spiramycine (macrolides); oxytetracycline (tetracyclines), amoxicilline

Table 2. Antibiotic resistance profile of *E. coli* strains.

Antibiotics used	Resistance % (number of strains)	Number of drugs families
AMP	7.69 (1/13)	1
TET	76.92 (10 /13)	1
MINO	76.92 (10 /13)	1
NAL	76.92 (10 /13)	1
GM	15.38 (2/13)	1
CIP	7.69 (1/13)	1
MINO/TET	76.92 (10/13)	1
NAL/CIP	7.69 (1/13)	1
NAL/MINO/ TET	76.92 (10/13)	2
NAL/MINO/ TET/AMP	7.69 (1/13)	3
NAL/MINO/ TET/GM	15.38 (2/13)	3

AMP: Ampiciline; TET: tetracycline; MINO: minocycline; NAL: nalidixic acid, GM: gentamycine; CIP: ciprofloxacine.

Table 3. Antibiotic resistance profile of *Salmonella.*

Antibiotics used	Resistance % (number of strains)	Number of drugs families
CIP	12.76 (1/8)	1
TET	87.5 (7/8)	1
MINO	75 (6/8)	1
NAL	87.5 (7/8)	1
NAL/CIP	12.76 (1/8)	1
MINO/TET	75 (6/8)	1
NAL/CIP/MINO/TET	12.76 (1/8)	2
NAL/MINO/TET	75 (6/8)	2

TET: Tetracycline; MINO: minocycline; NAL: nalidixic acid.

(betalactamines), Sulfamethoxazole and trimethoprime (sulfonamides) were detected in all analysed samples. While, tetracycline, doxycyline, ampicilline, oxacilline and penicilline G were not detected in the poultry litter analysed in this study.

In general, in all samples analyzed, ciprofloxacine had the highest rates with values ranging from 8.41 ± 2.03 mg/kg in litter from Songon to 4.56 ± 1.20 mg/kg in samples from Anyama. For areas of Bingerville and Yopougon, ciprofloxacine residues amounts were respectively 6.66 ± 1.5 and 6.61 ± 0.192 mg/kg of litter.

For erythromycine, the values ranged from 6.74± 1.20 to 5.36 ± 0.4 mg/kg of litter while for spiramycine the rates ranged from 5.01±0.61 to 4.44±0.88 mg/kg of chicken litter. The highest macrolides amounts were observed in samples from Yopougon and the lowest in litter from Bingerville.

Among antibiotics of tetracyclines classes, only oxytetracycline was detected in the analyzed litter with values of 1.57±0.5, 0.74±0.01, 0.74±0.006 and 1.01±0.01 mg/kg for samples from Yopougon, Songon, Bingerville, and Anyama respectively. Sulfonamide concentrations were 0.15±0.001, 0.05±0.002, 0.09±0.00 and 0.151±0.3 mg/kg for Trimethoprime and 1.02±0.1, 0.09±0.002, 1.18±0.3 and 1.02±0.2 mg/kg for Sulfamethoxazole for chicken litter from Yopougon, Songon, Bingerville, and Anyama respectively.

The lowest levels of antibiotic residues were observed for amoxicillin (betalactamines) with rates of 0.015±0.0003, 0.008±0.0001, 0.010±0.0001, and 0.010±0.0005 mg/kg of litter for the areas of Bingerville, Yopougon, Songon, and Anyama, respectively.

DISCUSSION

The goal in this study was to determine the contamination level of chicken litter from Abidjan poultry farms by antibiotic residues and MDR bacteria. Thus, we isolated *Escherichia coli* and *Staphyloccocus aureus* in 100% of poultry litter samples analyzed. While Salmonella spp. was isolated with the rate at 75 %. This contamination with these bacteria is not surprising, as they are naturally colonizing the intestine of poultry and can contaminate litter via feces (Shen et al., 2023; Eltai et al., 2020).

Generally, many species of bacteria are detected in poultry litter or in its manure around the world. Among the bacteria usually detected, *E. coli, Salmonella, Staphyloccocus, Campylobacter, Clostridium, Listeria,* and *Streptococcus* occur in levels exceeding those recommended in manure considered suitable for soil amendment (Kyakuwaire et al., 2019). These are highly pathogenic bacteria with generally health devastating effects in humans and livestock and the potential to widely spread in the environment. Since poultry litter is most often used in vegetable crops, these bacteria could potentially contaminate the vegetables and could be transmitted to the consumers.

Moreover, antimicrobial susceptibility testing showed high resistance patterns for *S. aureus* to erythromycine, tetracycline, clindamycine, trimethoprime and to tetracycline, minocycline, nalidixic acid for *E. coli* and *Salmonella* strains. The findings aligned with other reports, and this resistance pattern may result from selective pressure induced by the use of these antibiotics during breeding (Ngogang et al., 2021; Mund et al., 2017). The overuse of antibiotics is considered to be the key factor promoting the emergence, selection, and dissemination of antibiotic-resistant microorganisms in both veterinary and human medicine (Ungemach et al., 2006). As described in other studies, *E. coli* and *S. aureus* from litter in this study were found to be highly MDR suggesting use of many drugs during breeding (Ngogang et al., 2021; Goualie et al., 2020; Rahman et al., 2020; Bodering et al., 2017). This can also be explained by the fact that these bacteria are susceptible to antibiotic selective pressure and therefore development of resistance. As *E. coli* is considered to be resistance genes reservoirs, MDR *E. coli* found in the study indicates the risk of spread of resistance genes to other bacteria and increase of AMR both in human and animal (Ngogang et al., 2021; Kaushik et al., 2018).

Additionnaly, Atidégla et al. (2016) demonstrated that application of contaminated poultry manure to the crops in gardening sites may result in contamination of vegetables by fecal bacteria including fecal coliforms, *E. coli*, and fecal streptococci. As these bacteria can persist for several months in the environment, assessment of their presence and persistence on crops and soils following use of poultry manure will be relevant as this can reveal a hidden One Health threat, especially in these cases where no decontamination is performed prior to use of litter as fertilizer. Overuse of antibiotics does not only favor the risk of emergence and transmission of MDR bacteria, but it also poses a problem of food and environmental contamination with antibiotic residues that can create severe threats for humans, animals and the environment.

The analyses carried out on samples collected showed the presence of various antibiotic residues mainly ciprofloxacine, erythromycine, spiramycine, and oxytétracycline in poultry litter generally used as manure for soil fertilization without preliminary treatment in our country. The contamination of poultry litter by these antibiotic residues is probably due to the use of these drugs during breeding. Indeed, approximately 50 to 80% of the antibiotics consumed are directly excreted by the organism. So, antibiotic residues are generally detected in soil, some waste or water at sometimes high levels (Ben et al., 2019; Madikizela et al., 2017; Díaz-Cruz et al., 2008). On the other hand, it is estimated that the use of antibiotics worldwide is between 100,000 and 200,000 tons/year, and about 50% are used in veterinary medicine. This high consumption of antibiotics leads to an increase in antibiotic residues in the environment (Madikizela et al., 2017). Since poultry litter is usually use as soil fertilizer, the presence of these molecules in high concentration observed in this study could lead to a modification of soil microbial biodiversity (Li et al., 2008). Indeed, after exposure to a mixture of 16 antibiotics (concentrations between 0.005 and 1.5 µg/L), Proia et al. (2013) observed alterations in the composition in the soil bacterial community. In addition, Laverman et al. (2015) demonstrated a change in bacterial community structure exposed to vancomycin (1000 µg/L) in river sediments downstream of wastewater treatment plants. Another consequence of the presence of antibiotics in poultry litter used as fertilizer is the development of antibiotic resistance in the natural microbial communities of these habitats (Bengtsson-Palme and Larsson, 2016). Finally, the mixing of several drugs in poultry litter observed in this study could result in significant toxicity to soil organisms or potentially interact with each other with a greater effect (synergistic effect) on the environment (Guo et al., 2017). In addition, the modification of soil microbial biodiversity, we can also supposed that these antibiotic residues could contaminate crops and then unfortunately consumers.

Conclusion

The study found high contamination of poultry litter by *S. aureus, E. coli* and *Salmonella* spp. Many strains of *S. aureus* tested were MDR against at least three classes of antibiotic. The findings also indicate that the analyzed poultry litter is contaminated with various antibiotic residues. Since no treatment is generally performed before using poultry litter as manure, it could be a source of environmental and crop contamination with MDR bacteria and antibiotic residues. Thus, the results of this study indicate that the direct use of poultry litter as a fertilizer can pose a significant risk to the environment and health for farmers, local populations, and consumers of vegetables produced in the Abidjan area.

Therefore, it is urgent, first, to impose proper antibiotic use in the poultry sector to reduce the emergence of MDR in this sector, generally and particularly in waste such as chicken litter. The results of this study also emphasize the importance of applying biological treatments, such as composting, before using chicken litter for soil fertilization. Indeed, the use of properly composted poultry manure with selected microorganisms should help reduce the number of pathogens and the amount of antibiotic residues, thus avoiding the application of contaminated manure.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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