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Original article

Plasmid profiling analysis of some meropenem-resistant *Enterobacteriaceae* isolates from poultry farms in Ado Ekiti, Nigeria

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ABSTRACT

Background: The presence of antibiotic-resistant microorganisms in food animals especially poultry is of great concern globally. **Aim**: This research was carried out to detect and characterize plasmid carriage and profiles among members of *Enterobacteriaceae* from different poultry breeds especially broilers, layers, and noilers chicken in Ado Ekiti, Ekiti State, Nigeria. **Method**: A total of 93 rectal samples were collected and isolation and identification were done using standard microbiological techniques. The bacterial isolates were identified using DNA sequencing analysis. Antibiotic susceptibility testing was carried out using oxoid single disc polymyxin b, gentamycin, fosfomycin, nitrofurantoin, meropenem, and tigecycline. Plasmid profile and plasmid curing were also done to determine molecular weight and route of resistance whether plasmid or not. **Results**: A total of 7 bacterial isolates which include Br1 (*Pseudomonas monteilii*) and Br7 (*Escherichia coli*) from broilers. Cl29 (*Shigella flexneri*) CL33 (*Proteus mirabilis*), and CL33w (*Proteus mirabilis*) were isolated from layers, and Cn 3 and Cn14 (*Bacillus cereus*) were isolated from noiler in Ado Ekiti and they were resistant to meropenem. From the plasmid profiling analysis, it was revealed that all the isolates harbored plasmids. The plasmid sizes ranged from approximately 8000-10000bp and resistance was mainly plasmid-mediated. **Conclusion**: The findings of this study prove the consequences of antibiotic resistance and its usage among poultries are alarming as well as the relationship of plasmid with antibiotic resistance which necessitates proper surveillance of antibiotic usage in developing countries, especially among poultry farms.

Introduction

A variety of antibiotics are used to treat illnesses brought by bacteria because they kill or inhibit bacterial development [1]. For decades, the natural phenomenon of antibiotic resistance has been amplified by the considerable growth in antibiotic usage, particularly in poultry farms. When bacteria develop the ability to withstand antibiotics, the drugs intended to kill the bacterium become ineffective [1].

Due to health consequences brought on by resistant microorganisms, such as a rise in mortality, challenges with treatment, and prolonged hospital stays, antibiotic resistance has truly become one of the most significant worldwide health issues.

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Unexpectedly, several *Enterobacteriaceae* now exhibit meropenem resistance despite the fact that meropenem is one of the medications with effective broad-spectrum activity [2].

A sizable varied group of rod-shaped, Gram-negative bacteria that are naturally present in the mammalian gut as well as other habitats make up the family *Enterobacteriaceae* [2]. *Escherichia coli*, *Salmonella* spp *and Shigella* spp are a few examples of these helpful markers of food quality, sanitation, and contamination [3, 4]. A variety of enteric diseases, endocarditis and diarrhea, as well as infections of the respiratory tract, skin, soft tissues, urinary tract, joints, bones, eyes, and central nervous system are caused by the *Enterobacteriaceae* [4].

The majority of *Enterobacteriaceae* can produce resistance mechanisms mostly through the blockage of the efflux pump and the presence of resistance genes on plasmids. According to **Tilahun et al.** [5], plasmids are the primary vector in the acquisition and propagation of multi-resistant organisms, either phenotypically or genotypically. The most common mechanism of antibiotic resistance is the horizontal gene transfer of resistant genes. Plasmid-encoded resistance antibiotics include the majority of currently used clinically important classes of antibiotics [6]. The use of antibiotics to speed up the growth of poultry has been a major cause of antibiotic resistance, particularly for the *Enterobacteriaceae* which are a component of the intestinal flora [2]. This work used plasmid profile analysis and antibiotic susceptibility testing to identify the genetic relationship behind antibiotic resistance. This research was carried out to detect and characterize plasmid carriage and profiles among members of *Enterobacteriaceae* from different poultry breeds especially broilers, layers, and Noilers chicken in Ado Ekiti, Ekiti State, Nigeria.

Material and methods

This study was a cross section study that was carried out between January 2022 to August 2022 in Ado Ekiti, Ekiti State, Nigeria. A total of 93 samples were collected by using a sterile swab to collect samples from the rectum of poultry (48 layers, 20 noilers, and 25 broilers) on a selected farm in Ado Ekiti and inoculated in sterile peptone water and transported to the laboratory in an ice pack for analysis. Samples were inoculated on a sterile MacConkey agar and incubated at 37^{o}C for 24 hours. All the bacterial isolates were isolated and identified using proper microbiological, biochemical, and molecular methods [7].

Antimicrobial susceptibly test

In this study, the commercially available antibiotics Oxoid single disc was obtained from Oxoid (Basingstoke, Hampshire, UK) was used to determine antibiotics susceptibility testing. The antibiotics disc used include polymyxin B (200µg), gentamycin (30 µg), nitrofurantoin (300 µg), fosfomycin (100 µg), meropenem (10 µg) and tigecycline (15 µg) (12). Antibiotics susceptibility testing Kirby-Bauer Disk diffusion method was performed to determine the antibiotic susceptibility of all the bacterial isolates using the Mueller-Hinton agar according to the guidelines and recommendations of the clinical laboratory standard institute, 2021 [7,8].

Molecular identification by DNA sequencing

Molecular identification by DNA extraction, amplification, and sequencing methods of 16S rRNA using 27F and 1625R universal primer pair [7]. PCR preparation cocktail consisting of 10 µl of 5x GoTaq colorless reaction, 3 µl of 25mM MgCl2, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each **27F 5'- AGAGTTTGATCMTGGCTCAG-3' and - 1625R,** 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3units of *Taq* DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8µl DNA template was prepared [7]. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with a PCR profile consisting of an initial denaturation at 94°C for 5 min; followed by 30 cycles consisting of 94°C for 30s, 50°C for 60s and 72°C for 1 minute 30 seconds and a final termination at 72°C for 10 mins. And chill at 4^{o}C GEL [5]. The integrity of the amplified about 1.5Mb gene fragment was checked on a 1.5% Agarose gel run to confirm amplification using the buffer (1XTris Acetic and EDTA buffer). Two (2µl) of 10X blue gel loading dye were added to 4µl of each PCR product and loaded into the wells. The gel was electrophoresed at 120V for 45 minutes visualized by ultraviolet trans-illumination and photographed. The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using the manufacturers' manual while the sequencing kit used was that of Big Terminator v3.1 cycle sequencing kit. Bio- Edit7 software.

Sequences generated were identified using nucleotide BLAST on the NCBI database [7].

Extraction of plasmid DNA and plasmid profiling

To determine, whether the antibiotic resistance was plasmid-mediated or chromosomal, the isolates were subjected to a plasmid DNA extraction procedure and agarose gel electrophoresis.

Plasmid extraction

A 1.5ml portion of overnight culture was spinned at 12,000rpm for 1min using a micro centrifuge and the supernatant was decanted. A total of 300µl of TENS (25mM Tris, 10mM EDTA (Ethylenediaminetetraacetic acid), NaOH (0.1 Sodium hydroxide), and SDS (0.5% sodium dodecyl sulphate) were added and then mixed together until it became sticky. A portion of 150µl of 3.0M sodium acetate (pH 5.2) was added and the tube vortexes till it was completely mixed. The mixture was micro-centrifuged for 5min at 13,000rpm to pellet i.e. (small particles created by compressing the original) cell debris and chromosomal DNA. The supernatant was transferred into a fresh tube (Eppendorf tube) and then mix with 800µl ice-cold absolute ethanol and then centrifuged for 10min to pellet plasmid DNA. The supernatant was discarded and the pellet was rinsed twice with 1ml of 70% ice-cold ethanol and dried at 45^{o}C for 15min. The dried pellet was re-suspended in 20-40 µl of TE (Tris and EDTA) buffer and stored at 4°C for further use [7, 9].

Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA based on its molecular weight. The concentration of agarose used is dependent on the size of DNA to be separated but 0.8% agarose is basically used for plasmid DNA. A 0.8gram portion of agarose powder was mixed with 100mls of 1X TBE (tris, boric acid, and EDTA) buffer. The mixture was dissolved by boiling on a hot plate using a magnetic stirrer and later allowed to cool after which 10 µl (0.001ml) of ethidium bromide was added. The mixture was poured into an electrophoresis tank with the comb in place to obtain a gel thickness and avoid bubbles. It was allowed to solidify. The combs were removed and placed with the tray in the electrophoresis tank. 1X TBE (ris base, boric acid, and EDTA) buffer was poured into the tank to ensure that the buffer covered the surface of the gel. A 15 µl portion of plasmid DNA was mixed with 2 µl of loading dye and was carefully loaded onto the well created by combs. An electrode was connected to the power in such a way that the negative terminal was at the end where the samples were loaded. Electrophoresis was run at 60-100V until the loading dye (i.e. bromocresol purple) had migrated about three-quarters of the gel and the electrode was turned off. DNA bands were visualized and photographed using Bio-Rad, Mini-Sub Get GT. The molecular weight of unknown plasmid DNA was extrapolated using the band motilities in the gel [7,9].

Curing of plasmid DNA

Curing of the plasmid was done to determine whether or not a plasmid encodes a trait that codes for antibiotic resistance or multiple antibiotic resistance. Curing was done using the sodium dodecyl sulphate (SDS). The isolate that showed resistance to Carbapenem due to plasmid bands was subjected to plasmid curing. One gram of sodium dodecyl sulphate (SDS) was added to 100ml of nutrient broth. The solution was autoclaved at 121^{o}C at 15psi for 15min (the mixture was autoclaved without the isolate). An overnight culture of the sample was standardized according to 0.5 McFarland standard and 0.5ml from the standardized solution was pipette using a Pasteur pipette into the sterile 100ml nutrient broth. This solution was incubated at 37^{o}C for 24h. After incubation, the isolate will be re-inoculated into a sterile nutrient broth and incubated for 24h. The plasmid then be isolated and electrophoresed to check if the plasmid is cured or not [7,9].

Post-curing sensitivity testing

The plasmid-cured isolates were tested against those antibiotics to which they were previously resistant especially, meropenem. The diameter of zone of inhibition was measured using meter rule in mm and the zones were compared with Standard antibiotics chart [8]. This method was used to determine if the resistance was plasmid or chromosomally mediated

Results

A total of 7 bacterial isolates from poultry especially, broilers, layers, and noilers which are identified to be Br1 **(***Pseudomonas monteilii*) Br7*(Escherichia coli*) from broilers. Cl29 (*Shigella flexneri*) Cl33, (*Proteus mirabilis*), and Cl33w, (*Proteus mirabilis*) *were* isolated from layers, and Cn3 and Cn14 *(Bacillus cereus*) *were* isolated from noilers as presented in **table (1)** and resistant to meropenem.

Antimicrobial testing

All bacterial isolates were resistant to meropenem but highly sensitive to gentamycin, nitrofurantoin, fosfomycin, and tigecycline even though some are resistant to polymyxin B and especially *Shigella flexneri* from the poultry layer was only sensitive to gentamycin and tigecycline as presented in **table (2).** Only *E. coli* from broilers is sensitive to polymyxin, others are resistant

Plasmid profiling

To investigate the genomic relation behind antibiotic resistance, plasmid profiling was done for all the identified bacterial isolates. All the bacterial isolates from poultry's harbored single plasmids with molecular weight ranging from approximately 8000bp to 10000bp as represented in **plate (1).**

Plate 2 shows plasmid profile analysis of bacterial isolates from poultries after plasmid curing and it was observed that after post sensitivity curing all bacterial were sensitive after plasmid curing showing that resistance is mostly plasmid-mediated

Table 1. Bacterial isolates identified using DNA sequencing methods.

S/N	Sample code	Organism identified	Sample sources	Accession number
	Br1	*Pseudomonas monteilii*	Poultry broiler	ON715708
	Br7	*Escherichia coli*	Poultry broiler	ON715709
	Cl29	*Shigella flexneri*	Poultry broiler	ON715710
	Cl33	*Proteus mirabilis*	Poultry layers	ON715713
	Cl33w	*Proteus mirabilis*	Poultry layers	ON715714
	Cn3	*Bacillus cereus*	Poultry noilers	ON715715
	Cn14	*Bacillus cereus*	Poultry noilers	ON715716

Table 2. Antimicrobial susceptibility test of isolated bacteria

S/N	Isolates code	Organisms	PB (200) µg Polymyxin	Meropenem 10 µg	CN (30) µg Gentamycin	F (300) µg Nitrofuratoin	Fos(100 µg) Fosfomycin	Tgc(15 µg) Tigecycline
1	Br1 Broiler	*P. monteilii*	(R)	(R)	(S)	(S)	(S)	(S)
2	Br7 Broiler	*Escherichia coli*	(S)	(R)	(S)	(S)	(S)	(S)
3	Cl29 layer	*Shigella flexneri*	(R)	(R)	(S)	(R)	(R)	(S)
4	Cl33 layer	*Proteus mirabilis*	(R)	(R)	(S)	(S)	(S)	(S)
5	Cl33w layer	*Proteus mirabilis*	(R)	(R)	(S)	(S)	(S)	(S)
6	Cn14 noiler	*Bacillus cereus*	(R)	(R)	(S)	(S)	(S)	(S)
7	Cn3 noiler	*Bacillus cereus*	R)	(R)	(S)	(S)	(S)	(S)

R-Resistance, S- Sensitive

Plate 1. Plasmid profile analysis of bacteria isolated from poultry (Broiler Br, Layers Cl, and poultry noilers Cn. The plate has single plasmid bands with a molecular weight of 10,000bp (Cl33 and 8000bp for (Br1, Br7, Cl 29 ,Cn 14,Cn3)

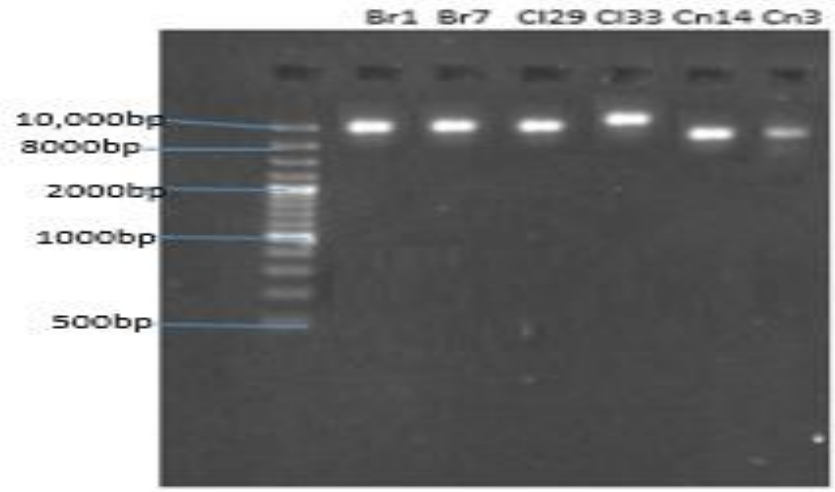
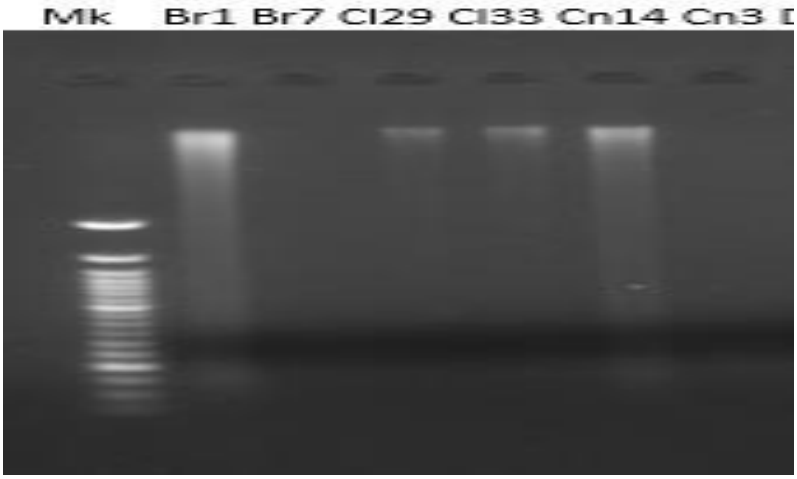


Plate 2. Plasmid profile analysis of bacterial isolates from poultry after plasmid curing



Discussion

Worldwide, Carbapenem-Resistant *Enterobacteriaceae* (CRE) infections pose a serious threat to the public's health and are linked to high morbidity and mortality [10]. Despite numerous measures to stop the spread of the diseases caused by CRE, the frequency of CRE varies greatly among various animals and geographical regions [10].

According to this study, all seven [7] bacterial isolates spp which include *Pseudomonas moneteilli, Shigella flexneri, E. coli, Proteus mirabilis,* and *Bacillus cereus* were resistant to meropenem, common carbapenems that are typically prescribed to patients who have infections that may be brought on by resistant bacteria. This was in line with the report of **Sanjida et al.** [11] in which several *Enterobacteriaceae* especially *E. coli* and *Proteus mirabilis* from farm animals particularly poultry birds, now exhibit meropenem resistance. This is alarming because meropenem was once highly effective but is now ineffective against some bacterial isolates [11].

Based on the result from this study, all the isolated bacteria were highly sensitive to especially nitrofuratoin, tigercyline, gentamycin and fosfomycin among others and this was in accordance with the report of **Sheu et al.** [12] that ancient antibiotics especially nitrofuratoin, tigercyline, gentamycin, polymyxin b and fosfomycin are still quite successful in treating bacteria, particularly meropenem-resistant strain. Although in this research, polymyxin B was not effective in the treatment of the bacterial as most were resistant to polymyxin. This suggest that polymyxin B might no longer effective in treating meropenem resistant bacteria.

In this study, the genomic relation behind the antibiotic resistance was done by plasmid profiling on all identified bacterial isolates which harbored single plasmid and the sizes ranges from 8000bp to 10000bp. This result was in accordance with the report of **Sheu et al, Szmolka et al.** [12,13] that most common plasmids have a size of approximately 8000bp to 12000bp.

The isolated bacteria were subjected to plasmid curing and post sensitivity testing to determine if resistance was plasmid mediated or chromosomally mediated. All the bacterial isolates that were resistant to meropenem became sensitive to meropenem after plasmid curing.

The seven (7) bacterial isolates with single plasmid bands have resistance mediated by plasmid and it acquired resistance which is in accordance with the report **Ekundayo** [7], **Muak et al.** [14], **Ngbede et al.** [16] which say that some selected *Enterobacteriaceae* especially *E. coli, Proteus mirabilis* which was also identified in this study from some animals have resistance to be plasmid mediated [15]. This suggests that the resistance genes can be highly transferrable from animal to humans. However, acquired resistance occurs because the bacterium has gained the ability to become resistant (usually via the acquisition of a plasmid containing the genes encoding the carbapenamase). Acquired resistance is more worrying because it can be spread by passing on the plasmid to other bacterial species which can cause severe infections e.g., *E. coli, K. pneumoniae* [15], and mortality from these resistant bacterial isolates is high [16].

Conclusion

Poultry animals have been found to be sources of the spread of antibiotic resistance, so there is a need for proper awareness and control of its spread through farm animals, especially poultry animals. Since most chicken breeders used antibiotics to promote growth and weight gain, the veterinary association in this region should step in to control the usage of antibiotics by chicken breeders. According to the study, the majority of resistance in this group suggests that resistance is primarily plasmid-mediated which can be highly dangerous to human health, therefore there is a need to create more awareness about the use of antibiotics among farm animals especially poultry birds. Additional research on the isolation of resistant bacteria from the habitats, feeds, and farm managers of the poultry bird will help determine whether the resistant bacteria are revolving, which will help determine how best to address the global problem of antibiotic resistance.

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Conflict of interest

Not declared.

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