Original Research Article

MOLECULAR CHARACTERIZATION OF RESISTANCE AND VIRULENCE GENES IN *Escherichia coli* ISOLATED FROM BATS (*Eidolon helvum*) FAECES IN OSUN STATE, NIGERIA

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ABSTRACT

Escherichia coli is one of the primary intestinal commensal organisms found in endothermic animals and hence, it is widely disseminated in the environment. A total of 36 (10.68 %) *Escherichia coli* was isolated from 101 faecal samples collected from straw coloured fruit bats (*Eidolon helvum*) faeces from three different major cities (Ile-Ife, Osogbo and Ilesa) in Osun State, Nigeria. The *E. coli* isolates showed a higher percentage antibiotic resistance to augumentin (83.33%), followed by cefuroxime (69.44%), ceftazidime (55.55%), amoxicillin (38.88%), and cefotaxime (33.33%), but relatively low to eterpenem, meropenem, and tetracycline (11.11%) and also nitrofurantoin (8.33%). Multiple antibiotic resistance to three or more antibiotics was recorded among the isolates in all the study locations. *Escherichia coli* (16.7%) were multiple antibiotic-resistant. Six (100%) of the multiple antibiotic-resistant *E. coli* posesses *MultiDHA* gene while 3 (50%) were positive for *TEM* gene. The virulence gene *eaeA* had the highest prevalence of 83.3% while the least was observed in PAPC (16.7%) and ISS (16.7%) but no biofilm production was observed in all the isolates. The resistance found across the three locations indicated that resistance genes can be transmitted to other animal and human through direct and or indirect contact. The antibiotic resistance profile and patterns as well as the antibiotic resistance genes detected indicated the possibility of cross transmission and spread of the resistance trait among the organisms with great

consequences in therapeutic management of infections resulting from such a source. The dendrogram shows that the isolates were genetically related across the three locations.

Keywords: Multiple antibiotic resistance, Resistance genes, *Eidolon helvum*

INTRODUCTION

Bats (Chiroptera) are taxonomically and ecologically diverse group of animal found in almost all habitats and trophic levels. There are about 1,300 species of bats documented [1]. Straw coloured Fruit Bat (*Eidolon helvum*) are one of the most important fruit bats species that form large colonies and they play a key role in seed dispersal, plant pollination and insect predation [2]. They have been implicated in epidemiologic cycles of several emerging and re-emerging zoonoses [3] and carriers of pathogenic agents that include more than 200 different types of viruses, fungi and pathogenic (enteric) bacteria mainly from the family Enterobacteriaceae [4]. Bats usually roost over human populated area and their indiscriminate release of excreta (guano) which are rich in diverse infections causing microorganisms could contaminate the environment and human (directly and indirectly), and therefore poses health risk. *Escherichia coli* is one of the primary intestinal commensal organisms found in endothermic animals and hence, it is widely disseminated in the environment. It can be responsible for a variety of diseases like diarrhoea [5]. The multidrug resistance *E. coli* is one of the major concerns in human health because of the ease of spreading the resistance as well as pose health challenges. Virulence factor expression is more common among certain genetically related groups of *E. coli* which constitute virulent clones within the larger *E. coli* population. In general, the more virulence factors a strain expresses, the more severe an infection it is able to cause.

MATERIALS AND METHODS

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Study Area

The study area are: Ile Ife {Obafemi Awolowo University, Ile-Ife lies between 7° 31' 14" N and 7° 31' 14".7612 N and longitudes 4° 32' 3.161"E and 4°. 32' 2. 591" E coordinates, Osogbo (Machine tools area, Km 8, Osogbo- Ikirun road) located at 7.835838°N 4.608353°E and Ilesa (Oba's Palaces Area, Ilesa) located at 7°37'0"N 4°43'0"E all in Osun State, South West, Nigeria.

Collection of faecal samples

One hundred and three faecal droppings of straw coloured fruit bats (*Eidolon helvum*) were collected randomly from various roosting places in three cities which include Ile-Ife, Osogbo and Ilesa in Osun State. The sample locations included Obafemi Awolowo University Ile-Ife, Nigeria, Machine tools, Osogbo and Oba's palace area, Ilesa. The samples were collected between 6.00 a.m and 7.00 a.m according to Akobi et al. [6]. The droppings were collected randomly from sterile white clothing into sterile bottle using a sterile swab stick and transported to the laboratory immediately in an ice to the laboratory for bacteriological analysis.

Sample Analysis and Isolation of bacteria

The faecal samples collected were immediately streaked on a molten sterile eosin methylene blue (EMB) agar plate, incubated at 37°C for 24 hours. Distinct colonies were picked and purified by successive and sub-culturing on freshly prepared nutrient agar plate, incubated at 37°C for 24. The isolates with characteristics metallic greenish sheen on EMB agar plate were presumptuously chosen as *E. coli* and isolates and were subjected to various biochemical tests including Gram's staining, catalase, Methyl Red, Voges-Proskauer test,

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citrate utilization, sugar fermentations, motility, indole, nitrate reduction, oxidase and urease activity. These were further confirmed by the Analytical Profile Index (API) 20E kit (bioMérieux, Inc., France). The isolates were stored on nutrient agar slant and kept in the refrigerator at 4^{o}C for further use.

Antibiotic susceptibility testing

The antibiotic susceptibility of the *E. coli* isolates was determined by Kirby-Bauer's disc diffusion method [7].

Each of the identified organisms (*E. coli*) was inoculated aseptically directly inside the test tubes containing nutrient broth and incubated for 24 hours at 37^{o}C. This was standardized using spectrophotometer at optical density of 540 nm to obtain 0.5 McFarland standards (10^{8}cfu/ml). The Standardized inoculum was finally seeded on sterile Mueller-Hinton agar plates (Lab M Ltd, UK) with the aid of sterile swab stick and allowed to dry for 5-10 minutes.

The antibiotic discs; single (Oxoid Ltd, Basingstoke, Hampshire, England) and combined (Abtek Biological Ltd, UK) of varying and specific concentrations were employed for the tests viz; cefotaxime (30 µg), ampicillin (10 µg), amoxicillin (3 µg), augmentin (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), cefuroxime (30 µg), ofloxacin (5 µg), cefixime (5 µg), gentamicin (10 µg), ciprofloxacin (5µg), nitrofurantoin (300µg), sulphonamethaxzole/trimethoprim (30 µg), meropenem(10 µg), and ertapenem (10 µg).

The discs were firmly placed on the surface of the culture plates using a sterile forceps and incubated in an inverted position at 37 °C for 24 hours. The diameters of zones of inhibition of each antibiotic on the bacterial isolates were measured by a transparent ruler to the nearest millimetre and compared with CSLI (2017) chart of interpretative zone as "Sensitive, Resistance and Intermediate resistance". Multiple antibiotic resistant isolates were considered as resistance to more than two classes of antibiotics.

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Molecular Characterization of Selected Multiple Antibiotic Resistant Isolates

The multiple antibiotic resistant *E. coli* across the three locations were selected. Extraction of DNA was done by boiling method. Bacterial isolate grown overnight was transferred into Eppendorf tube and spun down at 14,000 rpm for 2 minutes. The supernatant was discarded and 600 µl of pre- warmed extraction buffer Cetyl Trimethyl Ammonium Bromide (CTAB) was added to the pellet and it was incubated at 65^{o}C for 30 minutes. The sample was removed from the incubator and allowed to cool to room temperature and chloroform was added and mixed by inverting tubes for 15 minutes. Thereafter, the sample was spun at 14,000 rpm for 15 minutes and the supernatant was transferred into a new Eppendorf tube and equal volume of cold isopropanol was added to precipitate the deoxyribonucleic acid (DNA). The sample was kept in the freezer for 1 hour and later spun at 14,000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol. The sample was then air dried for 30 minutes on the bench. The pellet was re-suspended in 100 µl of sterile distilled water. The DNA concentration of the samples was measured on spectrophotometer at 260 nm and 280 nm and the genomic purity (1.8 - 2.0 µl) was determined.

Detection of resistance and virulence genes in *E. coli* isolates from bat from the three locations

Isolates were investigated for the presence of antibiotic resistance genes (*MultiDHA*, OXA-1, and *TEM)* and virulence genes (Biofilm, *PapC, ISS, eaeA*) by PCR. Polymerase Chain Reaction was performed in a total volume of 25 µl containing 2.5 µl of both the forward and the reverse primers, 12.5 µl master mix, 2.5 µl free water nuclease and 5 µl of the extracted DNA (as DNA template), then DNA amplification was carried out with the thermal cycler. The PCR products were electrophoresed in a 1.5% agarose gel and run at 80 V for 2 hours,

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stained with 1% ethidium bromide and the electrophoretic products were scanned with UV-trans-illuminator.

The primers used for the detection of resistance genes are presented in Table 1 while that for virulence genes are in Table 2

d. Random amplified polymorphic DNA (RAPD-PCR)

Amplification of genomic DNA was made on an Agilent cycler 2200 (Germany), using the arbitrary decamers [8]. The RAPD (OPB-12) primer. Amplifications of genomic DNA were performed in a total volume of 25 µl reaction mix containing 12.5 µl of Taq polymerase 2X, (10 mM Tris-HCl (pH 9.0), 25 mM KCl, 2 mM MgCl2, 0.2 mM of each dNTP), 0.5 ul of the random primer forward and reverse sequence each and 2 µl of DNA lysate. After completion of the PCR, RAPD fragments were separated electrophoretically on 1.5% agarose gels in 1X TBE buffer, stained with ethidium bromide, and photographed on a UV transilluminator using a digital camera. Deoxyribonucleic acid from each band was amplified with the same primer more than once, and the banding patterns were compared [9].

Cluster analysis and construction of phylogenetic tree

The presence or absence of each individual band of the DNA from RAPD analysis was recorded for each lane on the gel representing a given sample. The data obtained from the sequence alignment were used to plot a tree diagram using Molecular Evolutionary Genetics Analysis (MEGA) 5.0 software.

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Statistical analysis

The data obtained were analysed using SPPS Version 17. Descriptive and frequency statistics were generated to describe the characteristics of the study population. Analysis of variance (ANOVA) test was used to test the significance of association of the variables.

Table 1. Primers used for the detection of resistance genes in the selected multiple antibiotic resistant *E. coli* isolates

Primer	Seguence (5^1-3^1)	Amplicon Size (bp)	Annealing Temperature (^oC)	References
MultiDHA-F *MultiDHA*-R	TGATGGCACAGCAGGATATTC GCTTTGACTCTTTCGGTATTC	997	55	[10]
OXA-1-F- OXA-1-R	ATGAAAAACACAATACATATCAACTTC GC GTGTGTTTAGAATGGTGATCGCAT T	820	55	[11]
TEM-F *TEM*-R	GCGGAACCCCTATTT G ACCAATGCTTAATCAGTGAG	967	55	[10]

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Table 2: Primers used for the detection of virulence genes in the selected multiple antibiotic resistant *E. coli* isolates

Primer	Sequence (5^1-3^1)	Amplicon Size (bp)	Annealing Temperature (oC)	References
Biofilm-F	GATTCAATTTTGGCGATTCCTGC	225	48	[12]
Biofilm-R	TAATGAAGTCATTCAGACTCATCC			
PapC- F	GACGGCTGTACTGCAGGGTGGCG	328	60	[12]
PapC- R	ATATCCTTTCTGCAGGGATGCAATA			
ISS- F	GGCAATGCTTATTACAGGATGTGC	258	50	[12]
ISS- R	GAGCAATATACCCGGGCTTCC			
eaeA-F	GTGGCGAATACTGGCGAGACT	891	55	[13]
eaeA-R	CCCCATTCTTTTTCACCGTCG			

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RESULTS

The distribution of *E. coli* isolates recovered from bats faecal samples collected from the three cities (Ile-Ife, Osogbo and Ilesa) in Osun State is shown in Table 3. Thirty-six (10.68%) *Escherichia coli* isolates were recovered from the three locations**.**

Table 3. The distribution of E. coli isolates

Locations	**Samples (n)**	**Total no of *E. coli* (%)**
Ile-Ife	48	17 (47.2%)
Osogbo	23	11(30.6%)
Ilesa	30	8 (22.2%)
Total	**101**	**36 (10.68%)**

Table 4 depicts the percentage resistance of the *Escherichia coli* isolates to specific antibiotic and class of antibiotics. For Ife isolates, resistance was high to augmentin (94.12 %) and least percentage resistance (11.77 %) was recorded against capabenem, nitrofurans, sulphonamides and tetracycline. *Escherihia coli* isolates from Osogbo location were resistant to ceftazidime (90.91%), amoxicillin (81.82%) and cefuroxime and cefexime with 72.73% each. The least percentage resistance was observed in nitrofurans, sulphonamides and tetracycline as 18.18% each. The isolates from Ilesa location was 100% resistance to augmentin, cefuroxime (62.5%) and least to amoxicillin/clavulanic acid, penicillin and aminoglycosides as 12.5% each.

Table 5 shows the pattern of *E. coli* from bat faecal samples across the three locations in Osun State. Diversities of MAR patterns were observed with isolates showing resistance to ampicillin, cefuroxime, ceftazidime, augmentin and amoxicillin/clavulanic acid across the three locations.

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There was no statistically significant difference (P≥0.5) in the percentage antibiotic resistance across the three locations.

Table 4. Resistance of the *Escherichia coli* isolates to specific antibiotic and class of antibiotics

Class of antibiotics	**Specific antibiotics (µg)**	**Number of resistant isolates in each locations (%)**		
		Ile –Ife (n=17)	**Osogbo (n=11)**	**Ilesa (n=8)**
Penicillins	Ampicillin	5 (29.41)	4 (36.36)	1 (12.5)
Cephalosporins	Cefotaxime	4 (23.53)	0	0
(Cephems)	Cefuroxime	12 (70.59)	8 (72.73)	5 (62.5)
	Cefixime	0	8 (72.73)	0
	Ceftazidime	8 (47.06)	10 (90.91)	2 (25)
Beta-Lactamase	Augmentin	16 (94.12)	6 (54.55)	8 (100)
Inhibitor	Amoxicillin/ clavulanic acid	4 (23.53)	9 (81.82)	1 (12.5)
Carbapenems	Eterpenem	2 (11.77)	3 (27.27)	0
	Meropenem	2 (11.77)	3 (37.27)	0
Nitrofurans	Nitrofurantoin	2 (11.77)	2 (18.18)	0
Fluoroquinolones	Ciprofloxacin	0	0	0
	Oflaxacin	0	0	0
Sulphonamides	Sulphomethaxozole	2 (11.77)	2 (18.18)	0

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Tetracycline	Tetracycline	2 (11.77)	2 (18.18)	0

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Table 5. Multiple antibiotic resistance (MAR) pattern of *Escherichia coli* from bat faecal samples in Osun State

Isolates Location	Multiple Antibiotic Resistance pattern	Freq	No of MAR pattern	Overall
Ile Ife	AUG, CAZ, CRX	6	6	6
	AUG, AMC, AMP, CXM	2		
	AUG, AMC, AMP, CRX,	1		
	AUG, CAZ, CRX, CXM,	2		
	AUG, AMP, CAZ,CRX, ETP, MEM SXT, NIT, TE	1		
	AUG, AMC, AMP, CRX, ETP, MEM SXT, NIT, TE	1		
Osogbo	AMC, CAZ, CXM	2	11	8
	AMC, CAZ, CRX, CXM	2		
	AMC, CAZ, CRX, CXM, CTX	1		
	AUG, CAZ, CRX, CXM, CTX	2		
	AMC, AMP, AUG, CAZ, GEN	1		
	AUG, AMC, AMP, CRX, ETP, MEM NIT, TE	1		
	AMC, AMP AUG, CAZ, CRX, CXM, ETP, MEM SXT AMC,	1		
	AUG, AMP, CAZ,CRX, ETP, MEM SXT, NIT, TE	1		
Ilesa	AUG, CRX,	3	4	4
	AUG, AMP	3		
	AUG, CAZ, CRX,	1		

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Distribution of resistance genes in the multiple antibiotic-resistant *Escherichia coli*

The antibiotic resistance genes present in the multiple antibiotic resistant *Escherichia coli* isolates are shown on Table 4. *Multi DHA* -997bp gene was detected in all the isolates (100%) and *TEM* -964 bp (66.67 %.). However, *OXM* genes was not detected in any of the isolates profiled.

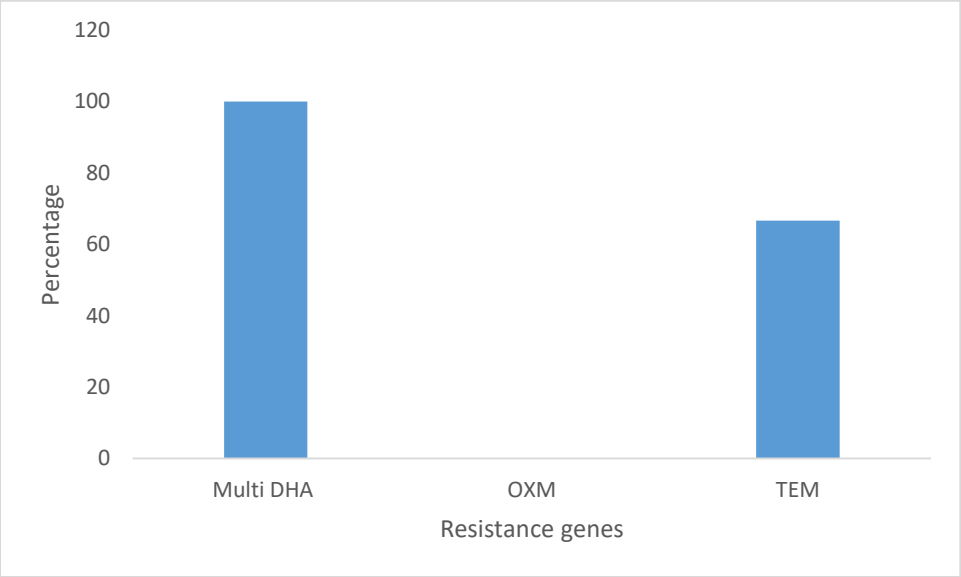

Figure 1: Distribution of resistance genes in the multiple antibiotic-resistant *Escherichia coli*

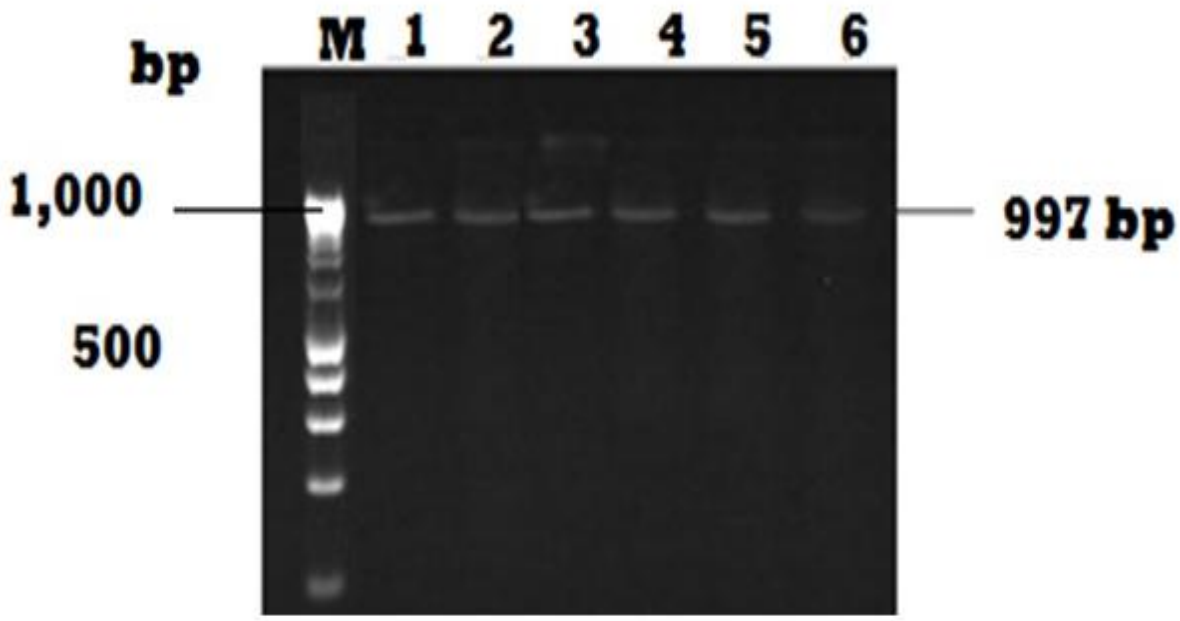

Plate 1: Agarose gel electrophoresis of *MULTIDHA* (997 bp) amplicons of the selected multiple antibiotic-resistant *E. coli* isolated from the bats faecal samples from the three locations in Osun State.

Lane M –DNA Ladder (100 bp).

Lanes 1 to 6 were amplicons from *E. coli* (IF11, IF26, OS55, OS83, OS62, IL42, respectively).

IF = Isolates from Ife

OS= Isolates from Osogbo

IL= Isolates from Ilesa

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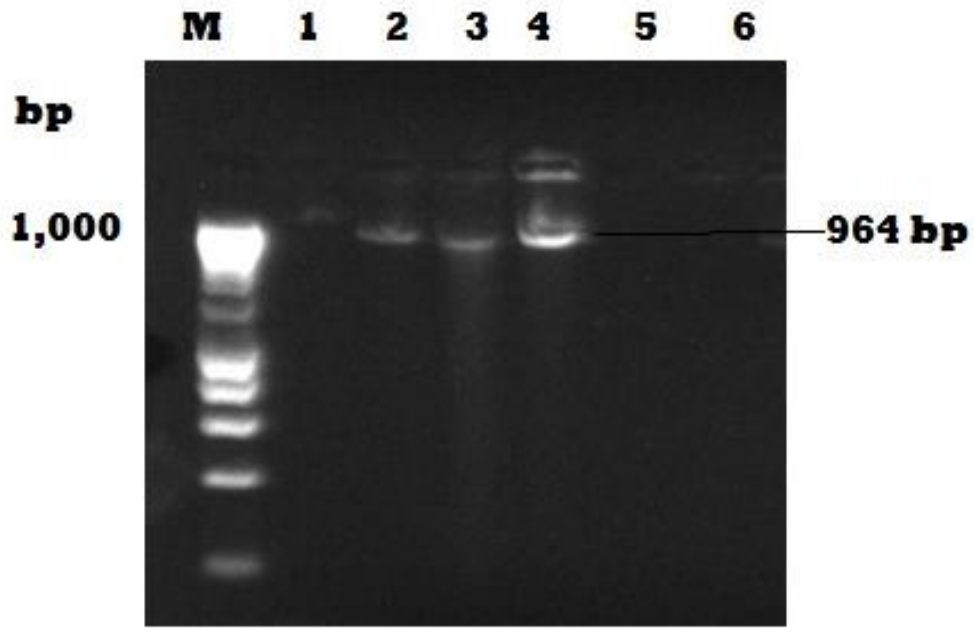

Plate 2: Agarose gel electrophoresis of *TEM* (964 bp) amplicons of the selected multiple antibiotics-resistant *E coli* spp isolated from the bats faecal samples from three locations in Osun State.

Lane M –DNA Ladder (100 bp).

Lanes 1 to 6 were amplicons from *E. coli* (IF11, IF26, OS55, OS83, OS62, IL42, respectively).

IF = Isolates from Ife

OS= Isolates from Osogbo

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IL= Isolates from Ilesa

Distribution of virulence genes in the multiple antibiotic-resistant *Escherichia coli*

The occurrence of e*aeA* genes was high (83.33 %), while *PAPC* and *ISS* genes were 16.67 %. However, non of the isolates tested negative for biofilm Production (Plate 3). The agarose gel electrophoresis of e*aeA* (891 bp) amplicons and Plate 4 shows the agarose gel electrophoresis of *ISS* (258 bp) and *PAPC* (328 bp) amplicons of the multiple antibiotic resistant *E. coli* from the bats faecal samples from the three locations in Osun State

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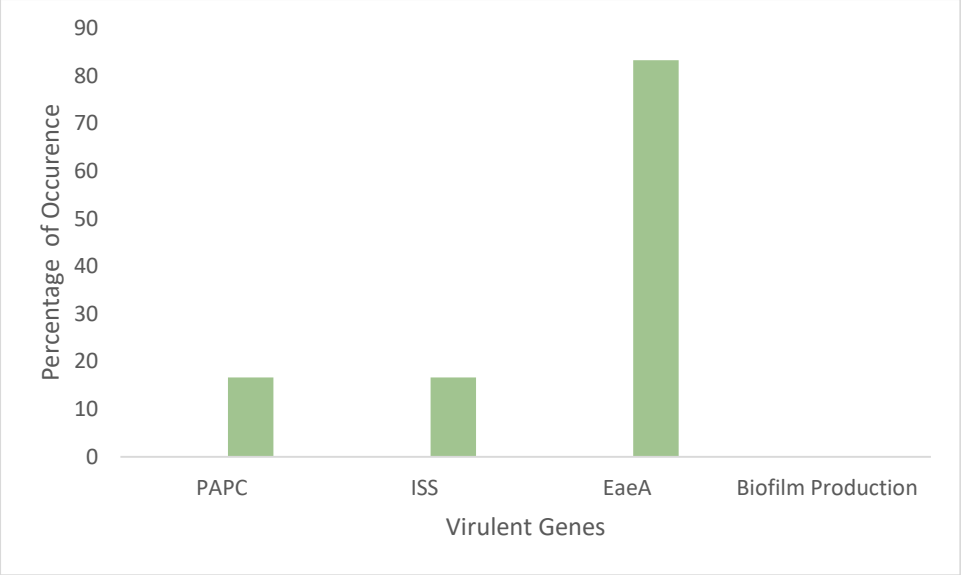

Figure 2: Distribution of virulence genes in the multiple antibiotic-resistant *Escherichia coli*

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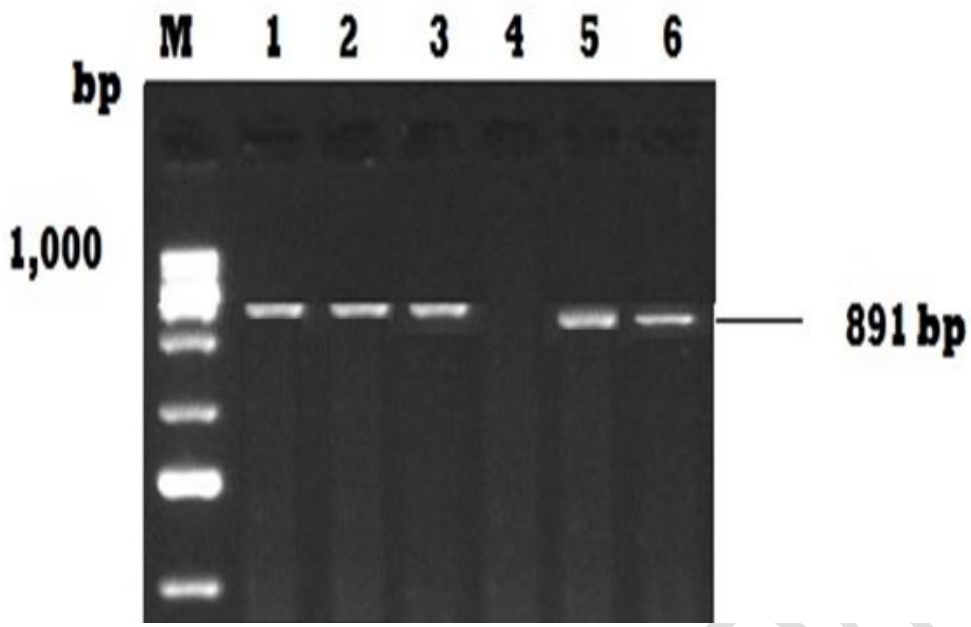

Plate 3: Agarose gel electrophoresis of *EaeA* (891 bp) amplicons of the selected multiple antibiotic-resistant *E. coli* isolated from the bats faecal samples from the three locations in Osun State.

Lane M –DNA Ladder (100 bp).

Lanes 1 to 6 were amplicons from *E. coli* (IF11, IF26, OS55, OS83, OS62, IL42, respectively).

IF = Isolates from Ife

OS= Isolates from Osogbo

IL= Isolates from Ilesa

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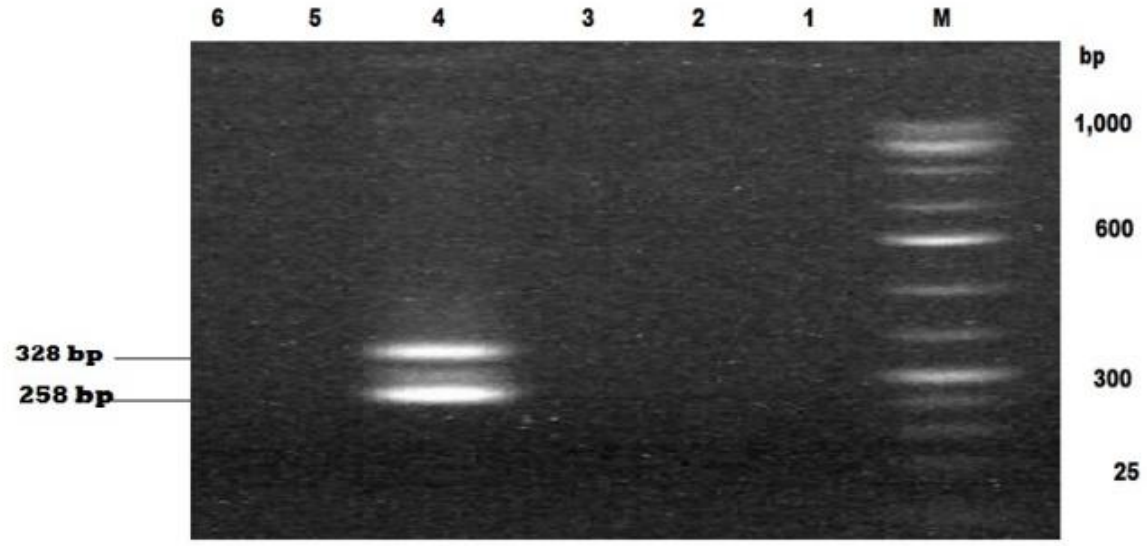

Plate 4: Agarose gel electrophoresis of *ISS* (258 bp) *PAPC* (328 bp) amplicons of selected multiple antibiotic-resistant *E. coli* isolated from the bats faecal samples from the three locations in Osun State.

Lane M –DNA Ladder (100 bp).

Lanes 1 to 6 were amplicons from *E. coli* (IF11, IF26, OS55, OS83, OS62, IL42, respectively).

IF = Isolates from Ife

OS= Isolates from Osogbo

IL= Isolates from Ilesa

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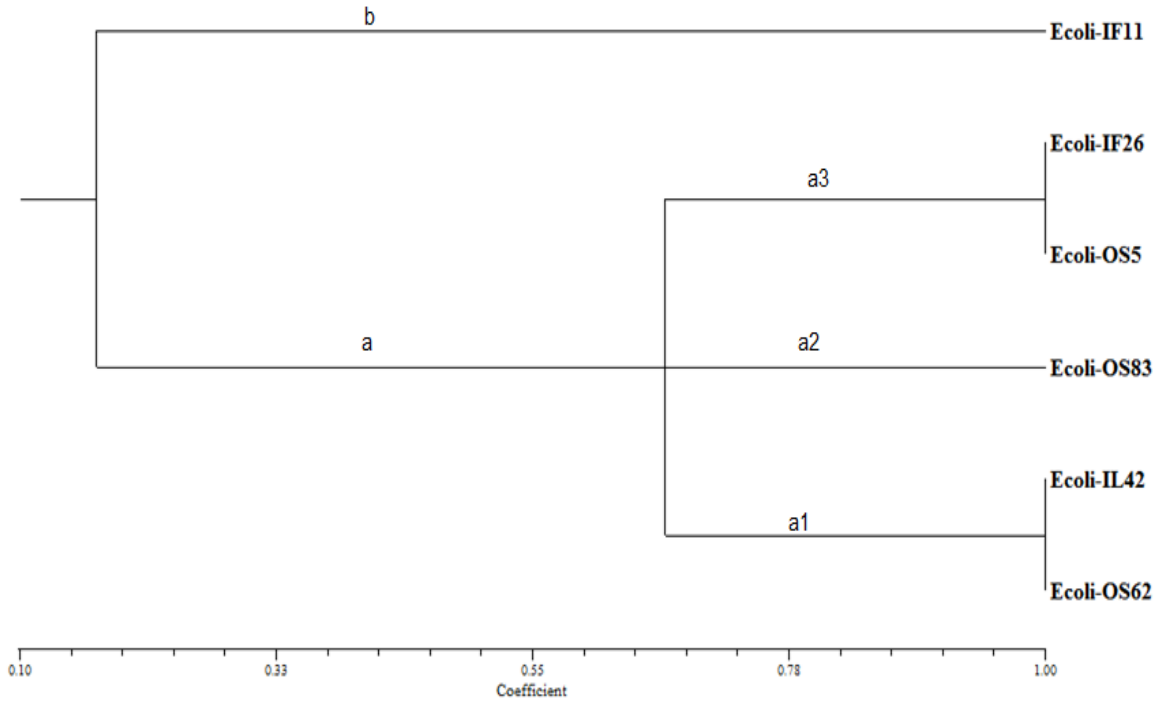

Fig 3: Dendogram of the selected multiple antibiotic resistant *E.coli* isolated from bat faecal samples from the three locations in Osun State.

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Isolates IF11, IF26, OS55, OS83, OS62, IL42, were selected antibiotic antibiotic resistant *E. coli* isolated from bats faecal samples from the three locations in Osun State.

IF= Isolates from Ile-Ife

OS= Isolates from Osogbo

IL= Isolates from Ilesa

Table 6 Virulence genes detected among multiple antibiotics resistant *E. coli* isolates obtained from faecal samples of straw coloured fruit bats

Virulence genes profiles detected by PCR	No. of isolates	Phylogenetic group			
		ai	a2	a3	b
EaeA	5	2	-	2	1
ISS	1	-	1	-	-
PAPC	1	-	1	-	-
Biofilm production	-	-	-	-	-

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DISCUSSION AND CONCLUSION

In this study, *Escherichia coli* had a prevalence of 47.2 %, 30.6 % and 22.2 % in Ile-Ife, Osogbo and Ilesa locations respectively. This percentage occurrence was relatively low compared to 50.5 % occurrence reported by [14] in zoonotic bacteria among illegally traded wild birds in Rio de Janeiro. The result of this present study can be compared to the work of

[15] which reported the presence of *E. coli* in the faecal samples of straw coloured fruit bats in Ile-Ife, Nigeria.

Considering the growing interest and effort in the study of antimicrobial resistance in wildlife, the information on bats is still limited especially in Africa. A few studies that were available [15] [6] recorded antimicrobial resistance in straw coloured fruits bats.

The flight capacity and ability to migrate from one region to another of bats, allow them to be more easily exposed to antimicrobials, increasing the risk of developing, changing or acquiring a new microbiota. The bacteria transmitted by bats are of a public health issue that is, even more difficult to eradicate and/or controlled [15].

The sensitivity pattern of the bats faecal *E. coli* Isolates from the three locations particularly ciprofloxacin and ofloxacin from the class Fluoroquinolones may be due to the non-frequent use in agricultural practice for animal feeds, and non-abuse of the antibiotics. Bacterial isolates showed high resistance of 94.12 %, 54.55 %, and 100 % to augmentin in Ile-Ife, Osogbo and Ilesa, respectively. This agrees with the study of [16] who reported a relatively higher (92 %) resistance to augmentin among the *E. coli* isolates from faecal samples among free range chickens, broilers and bats in Ile-Ife. Carbapenemases are β-lactamases with a wide hydrolytic spectrum. These enzymes inactivate almost all hydrolyzable β-lactams including the carbapenems as a unique, additional substrate. Carbapenemases are among β-lactamases from Ambler class A, B and D [17]. In the present study, eterpenem and meropenem were the antibiotics used in this class. The percentage resistance although low, but was significant across the three locations. In a study conducted by [18], the occurrence of antimicrobial resistance in *E. coli* strains isolated from bats in Mexico was much higher than in other mammals. The antibiotic resistance (*MultiDHA* and *TEM*) genes were the most predominant detected in *E.coli* from faecal samples of straw coloured fruit bat in the study

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areas**.** The presence of *TEM* and *MultiDHA* genes is suggesting a threatening horizontal gene transfer in our community.

The Virulence gene (*eaeA*) was the most predominant present in all the locations. One of the isolates harboured *PAPC* and *ISS* genes simultaneously [19] identified virulence genes from *Escherichia coli* isolated recovered from European free-tailed bat (*Tadarida teniotis*) in Portugal according.

Random Amplified Polymorphic DNA (RAPD) analysis revealed genetic relatedness among the selected multiple antibiotic resistant *E. coli* from the three different locations, therefore they probably originated from the same source. From this, it was revealed that there could be a transfer of bacterial isolates from one location to the other among the Straw coloured fruit bats *Eidolon helvum* which means that the infections caused by these bacteria can be transferred both vertically and or horizontally among the bats in different location across the studied geographical locations. To this effect, antibiotic-resistant bacteria as well as multiple resistant traits can be easily transferred across the geographic locations and even beyond.

The multiple antibiotic resistance profile and various MAR patterns observed among the isolates across the three studied locations calls for great concern as this may aid the spread of the resistance trait within and across species of bacteria in the locations with great health risk consequence.

This study concluded that straw coloured fruit bats (*Eidolon helvum)* are a potential carrier of multiple antibiotic resistant *Escherichia coli* which contain both resistance genes and virulence genes and could be a possible source of human infections through direct contact with the bat faeces as well as bat consumption. The genetic relatedness of the isolates is an indication of an easy transfer of zoonotic diseases

COMPETING INTERESTS DISCLAIMER:

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Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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