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Molecular characterization of extended spectrum β-lactamase-producing- Enterobacteriaceae from bats (*Eidolon helvum*) faeces in Osun State, Nigeria

Original Article

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Abstract:

Enterobacteriaceae from faecal samples of *Eidolon helvum* were isolated and identified biochemically then further confirmed using Analytical Profile Index 20E Kit. Antibiotic susceptibility testing and extended spectrum β-lactamase (ESBL) production using Mast Disc were carried out. Detection of *CTX-M* gene was done using polymerase chain reaction (PCR) employing appropriate primers. A total of 337 bacterial isolates was recovered from the studied locations consisting of forty-one species belonging to fifteen genera. Species of *Citrobacter*, *Enterobacter*, *Salmonella*, *Klebsiella* spp, and *Escherichia coli* were the most abundant and common to the three studied locations, with *Salmonella* spp being the most predominant. A total of 35.9% of the selected isolates was produced ELBS and 14.28% of the isolates harbours *CTX-M* gene. The study concludes that *E. helvum* in the study areas are reservoirs of Enterobacteriaceae and some harboured *CTX-M* gene which confirmed their pathogenicity with public health consequences.

Key words:

Eidolon helvum, Enterobacteriaceae, extended spectrum β-lactamase

Apstract:

Molekularna karakterizacija Enterobacteriaceae iz fecesa slepog miša (Eidolon helvum) u Osunu, Nigerija koje produkuju β-laktamaze proširenog spektra

Enterobacteriaceae su izolovane iz fekalnih uzoraka Eidolon helvum i biohemijski identifikovane korišćenjem API (Analytical Profile Index) 20E kita. Korišćenjem Mast diskova urađeno je i testiranje osetljivosti na antibiotike i testiranje produkcije β-laktamaze proširenog spektra (ESBL). Detekcija gena CTX-M je urađena je polimeraznom lančanom reakcijom (PCR) korišćenjem odgovarajućih prajmera. Na ispitivanim lokacijama utvrđeno je ukupno 337 izolata koji pripadaju 41 vrsti iz 15 rodova. Vrste Citrobacter, Enterobacter, Salmonella, Klebsiella spp, i Escherichia coli bile su najzastupljenije i česte za tri ispitivane lokacije, sa najčešćom Salmonella spp. Ukupno 35.9% odabranih izolata produkovalo je ESBL i 14.28% izolata nosilo je gen CTX-M. Istraživanje je zaključilo da su E. helvum rezervoari Enterobacteriaceae u istraživnim oblastima i da neki nose CTX-M gen koji potvrđuje njihovu patogenost i posledice po javno zdravlje.

Ključne reči:

Eidolon helvum, Enterobacteriaceae, β-laktamaze proširenog spektra

Introduction

Enterobacteriaceae consist of a large number of closely related bacterial species that inhabit the large bowel of man and animals, soil, water, and decaying matter (Hassan et al., 2011). These organisms are responsible for various infections including nosocomial or hospital-acquired infections that cause urinary tract and wound infections, pneumonia, meningitis and septicaemia, among others (Ruiz et al., 2002). Wild animals can serve as sources of various infectious diseases, including the majority of nosocomial or hospital-acquired and illnesses resulting from environmental contamination. Emerging infectious diseases are increasingly originating from wildlife. Different activities result

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into emerging infectious diseases of wildlife and they include:- alteration of an ecosystem, transfer of pathogens and vectors by human or other agents as well as advice in microbial genomic studies which reveals more about the infectious agents. The transmission of pathogens from wildlife to other animals and humans could be through direct or indirect contact (Rabinowitz et al., 2009). Wildlife although not directly exposed to antimicrobial agents can acquire resistance to clinically used antimicrobial agents through contact with the polluted environment, domestic animals and humans. Bat is a natural reservoir of deadly viruses, such as rabies, Ebola viruses and zoonotic bacterial pathogens, such as *Escherichia coli* and *Staphylococcus aureus* among others. (Oluduro, 2012; Akobi et al., 2012). Bat can easily spread multiple antibiotic resistant Enterobacteriaceae due to their versatile feeding and migratory capability. Antibiotics are used in the treatment of infections caused by Enterobacteriaceae. The active antimicrobial agents available for use against these organisms include; fluoroquinolones, β -lactams and aminoglycosides. Resistance to beta-lactams in Enterobacteriaceae is mainly conferred by β-lactamases. These enzymes inactivate beta-lactam antibiotics by hydrolysis. The most common ESBLs are SHV-, TEM-, CTX-M. The CTX-Ms are increasingly detected worldwide (Paterson & Bonomo, 2005; Pierre et al., 2020).

Materials and Methods

Study Area

The study area were selected cities where bats roost over a human-populated place in Osun State, Nigeria. These included: 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°50'9.0168'' N and 4°36'30.0708'' E and Ilesa (Oba's Palace Area, Ilesa) located at 7°37'0'' N and 4°43'0'' E (**Fig. 1**).

Sample collection

Faecal droppings of bats were collected randomly from various roosting places in Osun State, in the locations stated above. The samples were collected between 6.00 a.m and 7.00 a.m according to the method of Akobi et al. (2012) and transported to the laboratory immediately in an ice bag for bacteriological analysis.

Isolation of bacteria

The faecal samples collected were immediately streaked on prepared MacConkey agar plates and incubated at 37 °C for 24 hours for isolation of Enterobacteriaceae. Distinct colonies were picked and sub-cultured by successive streaking on freshly prepared MacConkey agar plates, incubated at 37 °C for 24 hours and a pure isolates were obtained. These were stored on nutrient agar slant and kept in the refrigerator at 4 °C for further use. All the isolates were identified using cultural, morphological, microscopic examination and biochemical tests following standard procedures and interpreted according to BERGEY's Manual of Systematic Bacteriology. Isolates were further confirmed using Analytical Profile Index (API) 20E test kit (BioMérieux, Inc., France).

Fig. 1. Map of Osun State and the study areas

Phenotypic detection of extended spectrum β-lactamase (ESBL) production

Selected multiple antibiotic-resistant Enterobacteriaceae isolates from faecal samples of bats were tested for ESBL production. This was carried out by using Mask disc comprising six antibiotics of ceftazidime (30 µg), ceftazidime plus clavulanic acid (30 µg), cefotaxime (30 µg), cefotaxime plus clavulanic acid (30 µg), cefpodoxime (30 µg), and cefpodoxime plus clavulanic acid (30 µg). The standardised inoculum of the isolates were inoculated aseptically on Müeller-Hinton agar plates. These discs were firmly placed on the surface of the culture plates aseptically using a sterile forceps and incubated in an inverted position at 37 °C for 24 hours. The diameters of inhibition zones of each antibiotic on the bacterial isolates were measured with a transparent ruler to the nearest millimetre. An increase in zone diameter greater than or equal to 5 mm in the presence of clavulanic acid from any or all the sets of MAST ID ESBL Detection Disc was considered to indicate the presence of ESBL in the tested organisms.

Detection of resistance gene (CTM gene) in selected ESBL-producing Enterobacteriaceae isolates by Polymerase chain reaction (PCR)

For the extraction of DNA, randomly selected ESBL-producing Enterobacteriaceae isolates grown overnight were transferred to 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 incubated at 65 °C for 30 minutes. The sample was removed from the incubator and allowed to cool to room temperature, chloroform was added and mixed by inverting tubes for 15 minutes. After that, the sample was spun at 14,000 rpm for 15 minutes and the supernatant was transferred into a new Eppendorf tube, afterwards equal volume of cold isopropanol was added to precipitate the DNA. The sample was kept in the freezer for 1hour 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 resuspended 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 was determined. The genomic purity was between 1.8 –2.0 µl for all the DNA samples.

CTX-M genes were profiled in the isolates by PCR using the primer CTX-M-F-ATGTGCAGYACCAGTAARGTKATGGC, CTX-M-R-TGGGTRAARTARGTSACCAGAA YSAGCGG (8). PCR was performed in a total volume of 25 µl containing 2.5 µl of both the forward and the reverse of the primers, 12.5 µl master mix, 2.5 µl nuclease free water and 5 µl of the extracted DNA (as DNA template), then DNA amplification was carried out with the thermal cycler. The reaction mixtures were amplified by denaturation for 4 minutes at 95 °C, 30 cycles at 95 °C for 30 seconds, annealing temperature at 55 °C for 60 seconds. The PCR products were electrophoresed using 1.5% agarose gel. It was stained with 1% ethidium bromide and run at 80 V for 2 hours, the electrophoretic products were scanned with UV- transilluminator.

Data Analysis

Data were analysed using SPSS software (version 20). Proportions and the actual number of ESBL-producing Enterobacteriaceae isolates was used to describe frequency outputs for categorical variables. Mean and standard deviation were used to describe continuous variables.

Results

In all, 337 bacterial isolates were recovered from the three locations which includes 142 (42.12%) isolates from Obafemi Awolowo University, Ile Ife, 84 (24.93 %) from Nigeria Machine Tools area, Osogbo and 111 (32.94 %) from Oba's Palace area, Ilesa all in Osun State, Southwest, Nigeria.

The identified Enterobacteriaceae belong to 15 genera comprising 41 different species. The bacterial species identified based on their various biochemical reactions to the API Kits include the following: *Citrobacter freundi, C. koseri, C. diversus, C. werkmanii, C. rodentium*; *Enterobacter aerogenes, E. intermedius, E. cloacae, E. aquatilis E. asburiae, E. sakazakii*; *Proteus vulgaris, P. mirabilis*, *Salmonella arizonae*, *S. bongori,* S. *typhi, S. enterica, S. choleraesuis*; *Klebsiella pneumoniae*, *K. oxytoca*, *K. ornithinolytica*, *K. planticola*; *Shigella sonnei*, *Sh. dysentery, Sh. flexineris*; *Serratia. liquefaciens*, *S. marcescens, S. plymutica, S. odorifera*; *Raoultella terrigena, R. ornithinolytica*; *Yersinia frederiksenii, Y. aleksiciae; Y. mollaretii*; *Escherichia coli, Erwinia amylovora*, *Edwardsiella ictaluri*, *Kluyvery ascorbate, Providenica rettgeri,* and *Rahnella aquatilis.*

Table 1 shows the distribution of the bacterial isolates in the bats faeces across the three studied locations. For Ile-Ife location, *S. arizonae* has the highest frequency of 24 (16.90%) followed by *E. coli* 17 (11.97%) and the least frequency of 1 (0.70%) were recorded in *E. sakazaki, E. hormaecei, K. planticola, K. ornitinolytica, R. aquatilis, R. terri-*

Table 1. The percentage of Enterobacteriaceae isolates in faeces of *Eidolon helvum* from the three locations

Bacterial isolates	Ile-Ife (n=142) (%)	Osogbo (n=84) (%)	Ilesa (n=111) (%)	Number of Isolates (n=337) (%)
Citrobacter freundi	3(2.11)	2(2.38)	5(4.51)	10 (2.97)
C. koseri	3(2.11)	2(2.38)	3 (2.70)	8 (2.37)
C. werkmanii	1(0.70)	-	-	1 (0.30)
C. rodentium	6(4.23)	-	-	3 (0.89)
C. farmer	2(1.41)	-	-	2 (0.59)
C. diversus	-	-	4 (3.60)	4(1.19)
Escherichia coli	17 (11.97)	11(13.10)	8 (7.20)	36 (10.68)
Enterobacter aerogenes	2 (1.41)	5(5.59)	6 (5.40)	13 (3.86)
E. aquatilis	-	1(1.19)	-	1 (0.30)
E. intermidius	3 (2.11)	2(2.38)	-	5 (1.48)
E. asburiae	12(8.45)	4(2.80)	5 (4.50)	21 (0.21)
E. cloacae	2 (1.41)	1(1.19)	6 (5.40)	9 (2.67)
E. sakazaki	1 (0.70)	2(2.38)	-	3 (0.89)
E. hormaecei	1 (0.70)	-	-	1 (0.30)
Edwardsiella ictaluri	3 (2.11)	-	-	3 (0.89)
Erwinia amylovora	-	-	1 (0.90)	1 (0.30)
Klebsiella pneumonia	10 (7.04)	10 (11.91)	11 (9.91)	31 (9.20)
K. planticola	1 (0.70)	-	3 (2.70)	4(1.19)
K. oxytoca	13 (9.16)	9 (10.71)	5 (4.51)	27 (8.01)
K. ornitinolytica	1 (0.70)	-	-	1 (0.30)
Kluyvery ascorbate	2(1.41)	1 (1.19)	1 (0.90)	4 (1.19)
Proteus mirabilis	5(3.04)	1 (1.19)	6 (5.40)	12 (3.56)
P. vulgaris	2(1.41)	1(1.19)	3 (2.70)	6 (1.78)
Providenica rettgeri	2(1.41)	-	-	2 (0.60)
Rahnella aquatilis	1(0.70)	-	-	1 (0.30)
Raoultella terrigena	1 (0.70)	-	-	1 (0.30)
R. ornithinolytica	3 (2.11)	-	-	3 (0.89)
Serratia plymutica	5 (3.04)	-	3 (2.70)	8 (2.37)
S. odorifera	2 (1.41)	-	2 (1.80)	4 (1.19)
S. liquefaciens	1 (0.70)	1 (1.19)	-	2 (0.60)
S. marcescens	3 (2.11)	-	2 (1.80)	5 (1.48)
Salmonella typhi	-	1 (1.19)	4 (3.60)	5 (1.48)
S. enterica	-	7 (8.33)	5 (4.51)	12 (3.56)
S. bongori	2 (1.41)	2 (2.38)	-	4 (1.19)
S. arizonae	24 (16.90)	14(16.67)	12 (10.80)	50 (41.56)
S. choleraesuis	-	1 (1.19)	-	1 (0.30)
Shigella flexineris	3 (2.11)	3(3.57)	3 (2.70)	9 (2.67)
S. sonnei	-	4(4.76)	4 (3.60)	8 (2.37)
Yersinia aleksiciae	1 (0.70)	1(1.19)	-	2 (0.60)
Y. mollaretii	1 (0.70)	-	2 (1.80)	3 (0.89)
Y. fredrikseni	3 (2.11)	-	-	3 (0.89)

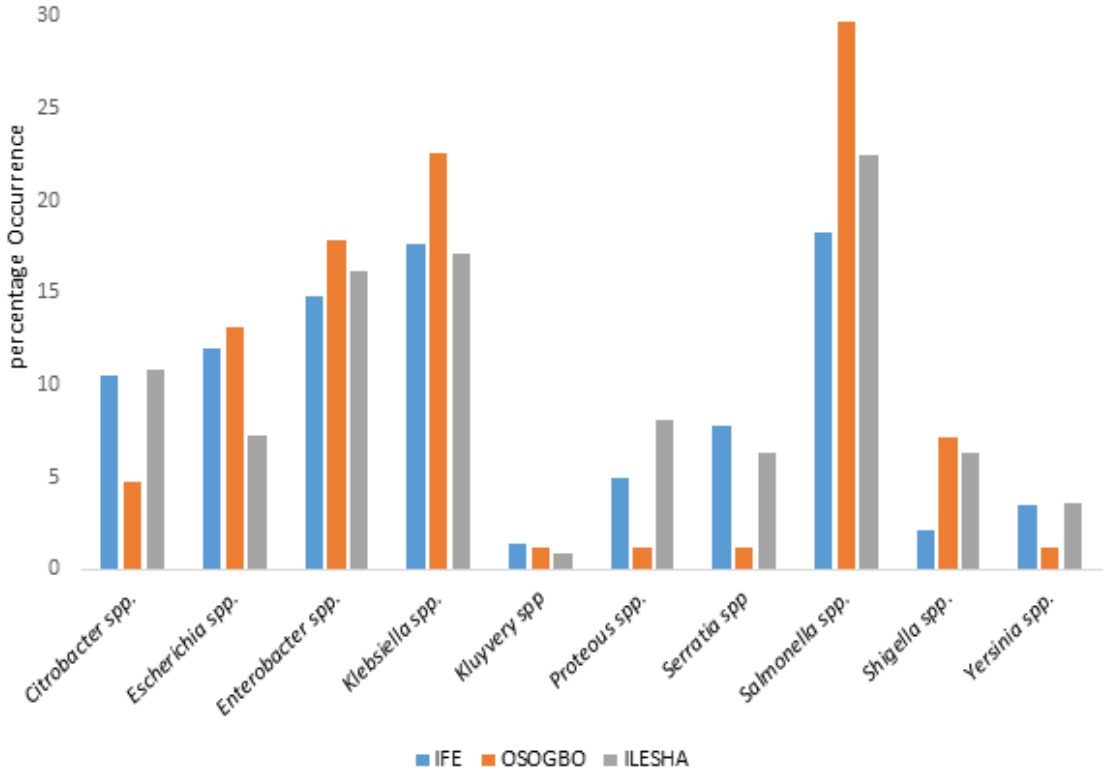

Fig. 2. Distribution of bacterial isolates common to the three locations (Ile-Ife, Osogbo and Ilesa)

gena, S. liquefaciens, Y. aleksiciae and *Y. mollaretii.* For Osogbo location, *Salmonella arizonae* has the highest frequency of 14 (16.67%) followed by *Escherichia coli* 11 (13.10%) and the leat frequency of 1 (1.19%) were recorded in *Enterobacter aquatilis, E. cloacae, Kluyvery ascorbate, Proteus mirabilis, P. vulgaris, S. liquefaciens, S. typhi, S. choleraesuis* and *Y. aleksiciae.* Also for Ilesa location. *S. arizonae* has the highest frequency of 12 (10.80%) followed by *K. pneumoniae* 11 (9.91%) and the leat frequency of 1 (0.9%) were recorded in *Erwinia amylovora* and *Kluyvery ascorbate.* In total, *S. arizonae* has the hig-hest frequency of 50 (41.56%), followed by *E. coli* 36 (10.68 %), *K. pneumoniae* 31 (9.20 %), *K. oxytoca* 27 (8.01%). *Salmonella* spp., *Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp., *Yersinia* spp. and *E. coli* were most abundant and common to the three locations as depicted in **Fig. 2**.

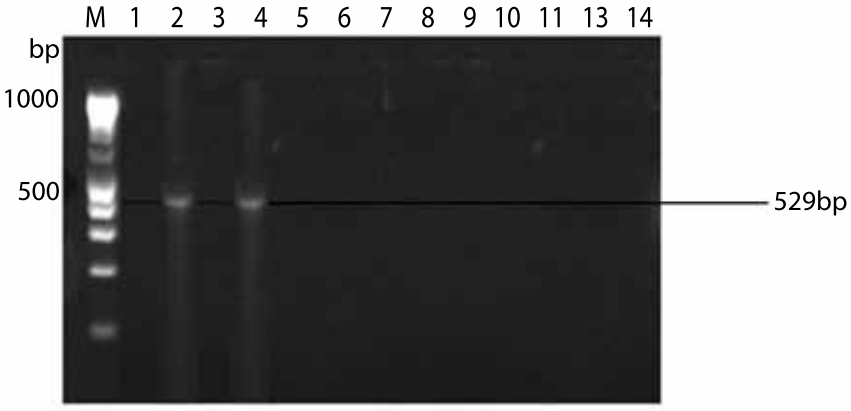

Fig 3: Agarose gel electrophoresis of *CTX –M* (529 bp) amplicons of the selected multiple antibiotics resistant *E coli* spp. isolated from faecal samples of *Eidolon helvum* from three locations

KEY

Lane M –DNA Ladder (100 bp).
Lanes 1 to 3 were amplicons from *E. coli*
Lanes 4 to 6 were amplicons from *Enterobacter sakazakii*
Lanes 7 and 10 were amplicons from *Salmonella arizonae*
Lanes 11 and 12 were amplicons from *Serretia liquefacience*
Lanes 13 and 14 were amplicons from *Citrobacter koseri*

Table 2 shows the occurrence of the ESBL-producing isolates in selected multiple antibiotic-resistant bacterial isolates from bat faecal samples. The ESBL-production by selected multiple antibiotic-resistant isolates using MAST Disc showed that 14 (35.90%) of the 39 selected antibiotic-resistant isolates were positive to ESBLs production.

Table 2. Occurrence of Extended-Spectrum β-Lactamase (ESBL) -Producing Isolates from faecal samples of *Eidolon helvum* from the Three Locations using Mast Disc

S/N	Isolates Name	Specific Isolate	Frequency
1	*Escherichia coli*	A84, B83, C42	3
2	*Enterobacter sakazakii*	A3, B29, A120	3
3	*Salmonella arizonae*	A5, B6, C15, B32, B44	4
4	*Serretia liquefacience*	A140, A69	2
5	*Citrobacter koseri*	A105, B3	2

*** Isolate Code**
Isolates from Ile-Ife: A84, A3, A120, A5, A140, A69 and A105
Isolates from Osogbo: B83, B29, B6, B32, B44, and B3
Isolates from Ilesa: C42 and C25

The *CTX –M* (529 bp) gene was detected in *E. coli and E. sakazakii* as shown in **Fig. 3**.

Discussion

Fifteen different genera comprising 41 different species of Enterobacteriaceae were recovered from the faecal samples of bats from the three study locations (Ile-Ife, Osogbo and Ilesa) in Osun State, Nigeria. The high diversity of isolates could be as a result of the ability of bats to travel far distance, which may result with feeding from different habitats. The Enterobacteriaceae recovered from the faecal samples of bats in this study were bacterial isolates that are significant in faecal samples of animals (both domestic and wild) across the world (Sader et al., 2018).

Salmonella arizonae has the highest frequency of 50 (41.56%), followed by *Escherichia coli* 36 (10.68), *K. pneumoniae* 31 (9.20%) and *K. oxytoca* 27 (8.01%).

Klebsiella pneumoniae had the highest occurrence of 10 (7.04%), 10 (11.91%) and 11 (9.91%) in Ile-Ife, Osogbo and Ilesa, respectively. Meanwhile, *K. oxytoca* had the next high percentage occurrence of 13 (9.16%), 9 (10.71%) and 5 (4.91%) in Ile-Ife, Osogbo and Ilesa location. These percentage occurrences were relatively low for *K. pneumoniae* (35.8 %) but relatively high for *K. oxytoca* (0.9%) when compared to the occurrence from wild birds (10).

Escherichia coli had a prevalence of 17 (11.97%), 11 (13.10%) and 8 (7.20%) in Ile-Ife, Osogbo and Ilesa locations, respectively. This percentage occurrence was relatively low compared to 50.5 % occurrence reported from wild birds (Carlos et al., 2016). The result of this study can be compared to the record of the presence of *E. coli* in the faecal samples of bats in Ile-Ife, Nigeria (Oluduro, 2012). *Salmonella* spp. and *Escherichia coli* have also been isolated from bat faeces in Trinidad according to Adesiyun (2009).

Ten (10) genera were common to the three studied locations while 13 species were common to the three studied locations, 13 species were common to two of the locations and 15 species were present in only one studied location.

Ile-Ife location has the highest diversity of Enterobacteriaceae with a total of 34 different bacteria species identified, Osogbo has a total of 21 different species while Ilesa has a total of 25 different species identified. The results show great diversity of Enterobacteriacea in the studied area, although statistical data indicate that there is no significant difference in the occurrence of isolates across the three locations.

Bats are unique with their ability to fly long distance (Calisher et al., 2006). Therefore they can migrate from one geographical location to another. This capability makes it easy for them to acquire new microorganism either from the environment, contact with one another or other animals which aid the resistance of their microbial commensals to antimicrobial agents. The antimicrobial resistance among these bacteria leads to ineffective treatment of the infections caused by these organisms. Various mechanisms of drug resistance have been attributed with the production of beta- lactamases which leads to resistance. The increase in the prevalence of ESBL-producing isolates globally increases the burden of treating infectious disease especially in developing countries with poor resources and weak health management system.

In the recent decade, ESBL-producing Enterobacteriaceae has resulted in an increase of resistance to β-lactam antibiotics leading to challenges in the management of infections caused by these bacteria (Cantòn & Coque, 2006).

In this study, 35.9% of the selected multiple resistant isolates were positive to (ESBLs) produc-

tion. It is an indication that substantial amount of the multiple resistant isolates were ESBLs producers. The reason may be that the third-generation cephalosporins are commonly in use over a long period in the studied areas and might have been abused, therefore leading to the acquisition of resistance mechanisms by the organisms. The decreased susceptibility of these antimicrobial could also be as a result of the production of ESBL and AmpC β-lactamase.

Two of the isolates profiled harboured *CTX-M* gene (*E. coli* (B83 from Osogbo) and *Enterobacter sakazakii* (A120 from Ife)). Although the CTX-M family of ESBLs has been reported in Germany since 1989 (Doi et al., 2017) it has now spread to different parts of the world. The CTX-M enzyme has been found in *E. coli* and *Klebsiella* spp., more frequently then in other Enterobacteriaceae species (Bush, 1989). Wildlife are not directly exposed to clinically used antibiotics, but they could have contacted it through environment (sewages and animal manure). Resistance could also be gained through feeding on some materials that contain antimicrobial substances in nature, like plants, but this still remain unclear. Due to the ability of migratory birds to travel far distance, they can carry antibiotic-resistant bacteria from one continent to another leading to global transmission of bacterial resistance among bats. Direct contact with faeces of wildlife and indirect contact through food, water and air is an important risk factor for the dissemination and transmission of pathogens or antimicrobial resistance from animals to both other animals and humans.

Conclusions

Currently, no studies have described the prevalence of ESBL-producing Enterobacteriaceae in faecal samples of *Eidolon helvum* in Nigeria, hence the study is novel. Although much has not been done on the probable source of contamination, however the result of this study indicates that bat is a source of ESBL-producing Enterobacteriaceae and hence prevention need to be taken since they roost over human-populated area and release their faeces indiscriminately.

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