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OCCURRENCE OF CARBAPENEM-RESISTANT ENTEROBACTERIACEAE (CRE) ABHORRING BLA IMP, BLA KPC AND BLA OXA-48 GENES RECOVERED FROM READY -TO- DISPOSE POULTRY WASTES IN OSOGBO, OSUN STATE

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The uncontrolled use of antimicrobials in animal husbandry such as poultry for prophylaxis reasons is to increase output for monetary gains which have led to several acquired community infections with high mortality and morbidity rates. This study is however aimed at investigating the occurrence, prevalence, and molecular detection of carbapenemase genes of carbapenem-resistant Enterobacteriaceae (CRE) bacteria isolated from poultry liters in Osogbo, Osun State Nigeria. Twenty ready-to-pack poultry waste samples were plated for the isolation of Enterobacteriaceae on MacConkey and Brilliance *Escherichia coli* agar. Disc diffusion method was employed for screening carbapenem resistant isolates while resistant genes were detected using carbapenem resistant gene primers. Forty-six Enterobacteriaeceae were isolated namely: *Kluyvera ascorbate* (28.3%), *Klebsiella oxytoca* (10.9%), *Citrobacter koseri* (8.7%), *Klebsiella aereogenes, Raoultell (Klebsiella) planticola, Serratia nematodipila* (6.5%), *Trabulsiella laguamensis, Salmonella* spp. *(diarizonae), Proteus vulgaris, Citrobacter farmeri* (4.3%), of which *Edwardsiella tarda, Providencia rustigianii, Citrobacter freundii, Enterobacter cloacae, Cedecea lapagei, Serratia liquefaciens* and *Dickeyachry santhemi* was (2.2%) respectively. 52.2% of the isolates were carbapenem resistant while 92% of the CRE had bla IMP genes. Only one CRE (*Trabulsiella guamensis*) was positive to bla KPC gene while four 16.6% of the isolates namely: *Citrobacter koseri, Raoultell (Klebsiella) planticola, Serratia nematodipila, Citrobacter freundiu* and *Citrobacter koseri* were positive to blaOXA-48 gene. Therefore, this study infers the high prevalence of CRE isolates in poultry litters as a public health concern for the indiscrimate use of carbapenem.

Keywords: carbapenem-resistant Enterobacteriaceae (CRE), Brilliance *E. coli* medium, disc diffusion, prophylaxis, prevalence

INTRODUCTION

The indiscriminate use of antibiotics in livestock and animal husbandry have steered the frightening rises in antimicrobial resistance in poultry birds (1). Various known genera of the Enterobacteriaceae have been documented to cause numerous infections with narrow treatment options. The members of the multi-drug antibiotic resistant species of Enterobacteriaceae includes *Citrobacter, Enterobacter, Klebsiella, Escherichia, Morganella, Salmonella, Shigella, Yersinia, Proteus, Serratia, Raoultella, Pantoea, Leclercia, Hafnia, and Kluyvera.* According to another study, microorganisms isolated from fecal contaminated environments are more prone to attaining resistance to common antimicrobial (2), Thus, scientist have engineered their research towards the monitoring of these multi-drug resistant organisms and mechanisms of their resistance (3, 4). Microorganism are ubiquitous in nature; hence they can also be isolated from poultry litters. In agriculture, poultry litter is the combination of poultry excreta which absorb moisture, dilute fecal materials, spilled feed, feathers and materials used for beddings (such as sawdust, wood shavings and rice hull) in poultry operations which are sources of air and soil pollution that can also harbor microorganisms (5). These microorganisms may contain pathogenic bacteria such as *Enterococci*, *Staphylococci* and Enterobacteriaceae and among others (6). Poultry litter quality and composition is of key importance in the general performance of the poultry birds as they can serve as sources of zoonotic infectious agents that affect the growth of the birds (7). If litters are not kept at an acceptable moisture level, high microbial load and unhygienic growing conditions may emerge, producing unpleasant odors, insects and rodent infestations which have negative effect in the environments and the people (8), solid feathers, footpad and blisters also promote the spread of pathogenic bacteria and molds (9). Ammonia concentration due to foul odor have also been documented to be implicated in the cause of burn and tear to the human eyes, increase in the incidence of breast blisters, skin burn and bruising. Indiscriminate drug usage also fosters antimicrobial resistance since the persistent ingestion of drugs against microbial infections leads to developed resistance of the microorganism. According to other report, the gut of poultry birds can be a reservoir for E. coli, which can further be transmitted to human by contact with the poultry wastes or contaminated poultry feeds (10).

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Antimicrobial resistance can spread from animals to humans and also from humans to animals through the direct transference of resistant bacteria or when resistant genes found in zoonotic microorganisms via diverse means to humans (4, 11). Antimicrobial resistance makes treatment of zoonotic infections challenging, and sometimes very difficult. However, the detection of new effective antimicrobial agents has been subdued by antimicrobial resistance. A previous report on antibiotic resistance documented that the current antibiotics used in treating zoonotic infections in man and animal may no longer work in the next 5 to 10 years (1). Hence, the need for rigorous efforts in the quest for alternative antimicrobials therapies that could be used as effective treatment against multi-drug resistant organisms that are also capable of transmitting resistance markers to other bacteria in their environment.

Carbapenem antibiotics are generally considered to be the most potent group of antimicrobial agents to multi-drug resistant, extended-spectrum β-lactamase and AmpC-producing Enterobacteriaceae with proven efficacy in the treatment of patients with severe bacterial infections, including those caused by other antimicrobial-resistant strains (12). The recent increase in the rates of carbapenem-resistant Enterobacteriaceae (CRE) among poultry litters and birds is of public health concern (13). This high surge in CRE is mostly driven by the emergence and spread of carbapenemase enzyme, a specific group of beta-lactamase that are capable of hydrolyzing carbapenems (14).

MATERIALS AND METHODS

Sample collection: A total of 20 poultry litters samples from ready-to-dispose poultry beddings were collected from twenty different poultry farms situated in Osogbo, Osun state, Nigeria at two months' interval from the first sampling time between January and July 2021. This poultry litters served as manure on the agricultural land by farmers. 10-20 g of samples were collected using sterile spatula by missing the poultry bedding, chicken waste and feaces in to sterile labelled universal bottle, stored in coolers containing ice packs and conveyed immediately to the laboratory further processing.

Bacterial identification: 1g of each poultry litter sample was weighed into 10 ml of sterile Ringer solution in test tubes and mixed properly using a vortex mixer at 1000 rpm. The mixtures were serially diluted in 9 ml of sterile Ringer solution and 100 µl was taken from 10^2 and 10^4 dilution factors. These were aseptically plated on Petri plates using spread plate method on MacConkey and Brilliance *E. coli* agar. Plates were incubated at 37ºC for 24 - 48 hr. Isolates were identified using standard phenotypic and biochemical methods according to the Bergey's Manual of Determinative Bacteriology (9^{th} Edition) characterizations. Results were interpreted using the ABIS online Microbiology software for bacterial identification (15, 16).

Phenotypic Characterization of CRE isolates: The pure cultures of bacterial isolates were screened for carbapenemases using (17) the MacConkey meropenem supplemented medium methods. Standard discs of ertapenem and imipenem (10 µg, Oxoid, England) and were used, following the CLSI. Antibiotic discs were placed on the surface of inoculated Mueller Hinton agar (MHA) plates using sterile forceps. Discs were placed at about 30 mm apart and incubated for 24 hr at 37ºC. Zones of inhibition (mm) were read and recorded after the incubation time. Isolates that showed a zone of inhibition ≤ 21 mm in diameter for meropenem and/or ertapenem were considered as suspected carbapenemase producers. Escherichia coli ATCC 25922 was used as a control. All Enterobacteriaceae isolates which were found resistant to meropenem (10 µg) and/or Ertapenem (10 µg), either alone or in totality were subjected to CRE confirmatory testing using modified Hodge test [15 and 17]. Pure isolates were streaked on 1µg/ml meropenem supplemented MacConkey agar plates as described by (18) and incubated at 37ºC for 24 hr. Carbapenem resistant K. pneumoniae grew while Carbapenem susceptible K. pneumoniae did not grow.

Modified Hodge Test: Using the methods described by (15, 17 and 19), a 1/10 dilution of 0.5 McFarland suspension inoculum of a control strain of *E. coli* ATCC 25922 was spread plated on MHA plates and 10 µg of imipenem disc was placed at the center of the plate. 0.5 McFarland standardized test isolates were streaked touching the antibiotic disc to the edge of the Petri plate, incubated at 37ºC for 18-24 hr. Test organisms were streaked together with the two quality control organisms Klebsiella pneumoniae ATCC BAA-1705 MHT positive and K. pneumoniae ATCC BAA-1706 MHT negative in a straight line from the edge of the imipenem disc. The appearance of a clover leaf type indentation at the intersection of the test organism and Escherichia coli ATCC 25922 was recorded as positive.

Antimicrobial Susceptibility Testing: Antibiotic susceptibility pattern of isolated organisms was screened using Kirby-Bauer modified disc diffusion method. 18-24 hr old pure cultures were inoculated into 5 ml of sterile Ringer's solution to give a turbidity equivalent to 0.5 McFarland standards. Sterile cotton-tipped applicators, one per isolate, was inserted into each inoculum tube, and swabbed onto the entire surface of MHA plates creating a lawn on the Petri plates. The antibiotics used in this study includes: oxacillin (1 µg), cefepime (30 µg), azithromycin (15 µg), kanamycin (30 µg), aztreonam (30 µg), colistin sulfate (10 µg), tigecycline (75 µg) and doripenem (10 µg). An 8-place disc dispenser was used to set the discs aseptically on the on the surface of the inoculated MHA plates and incubated at 37±2°C for 18-24 hr. Clear zones were examined visually after the incubation time, and the diameter of each zone of inhibition was recorded to the nearest mm. Results were interpreted as sensitive (S) and resistant (R) using the EUCAST breakpoint table vs 12.0 (EUCAST, 2022). Resistance to ≥ one antibiotics in ≥ 3 antibiotic classes were used as the standard for reporting an isolate as multidrug resistant, while multiple antibiotic resistance indices (MARI) were evaluated using established methods (16).

DNA Extraction and amplification of Carbapenemase producing organism: DNA extraction was done using the thermal lysis method as described by (21) with slight modification. Single colony of pure isolates was inoculated in to sterile 2 ml nutrient broth and incubated at 37ºC for 24 hr after which1 ml was taken and centrifuged at 12,000 rpm for 15 min. The pellets were collected and resuspended in to 200 µl TBE buffer, vortexed properly and boiled in a water bath for 10 min followed by another centrifugation at 12,000 rpm for 15 min. The supernatants were transferred into sterile Eppendorf tubes to determine their concentration and DNA purity using a (UV-Vis Thermo Scientific TM Nanodrop Life Spectrophotometer, (Model S-22, Boeco, Germany)

Previously described methods for amplification and detection of carbapenem encoding genes namely blaKPC, blaNDM, blaVIM, blaIMP and blaOXA−48 in the Enterobacteriaceae was employed in this study (2, 15, 22). The primers used is presented in Table 1 below while PCR was done using Master cycler gradient thermocycler with a final volume of 25 µl master mix consisting of 12.5 µl 2X universal PCR master mix, 2 µl of primers (10 µM) each of forward and reverse primers, 5.5 µl nuclease-free water and 5 µl of DNA template. The PCR protocol include: initial denaturation at 95°C for 10 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at appropriate temperature for each carbapenemase genes as depicted in table 1 for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min. Amplicons were visualized on 1.5% agarose.

RESULTS

Forty-six (46) Enterobacteriaceae was isolated from the twenty (20) ready to pack poultry waste samples. Presumptively identified organisms were: *Kluyvera ascorbate* (13/46. 28.3%) *Klebsiella oxytoca* (5/46.10.9%), *Citrobacter koseri* (4/46. 8.7%), *Klebsiella aereogenes, Raoultell (Klebsiella) planticola, Serratia nematodipila* (3/46. 6.5%), *Trabulsi ellaguamensis, Salmonella* spp. *(diarizonae), Proteus vulgaris, Citrobacter farmer* (2/46. 4.3%) *and Edwardsiella tarda, Providencia rustigianii, Citrobacter freundiu, Enterobacter cloacae, Cedecealapagei, Serratia liquefaciens, Dickeya Chrysanthemi* were (1/46. 2.2%) as shown in figure 1. Table 2 revealed the multiple antibiotic resistance pattern of the poultry litter isolates to commonly used antibiotics like aztreonam, cefepime, ceftazidime and doripenem. Twenty-four (52.2%) of the isolates were carbapenem resistant as shown in Figure 2 while twenty-two (92%) out of the twenty-four CRE isolated banded at base pair of 488 for bla IMP.

Table 1: The PCR Oligonucleotide sequence for amplification of CRE organisms used in this study.

Target Gene	Oligonucleotide sequence (5'-3')	Amplicon size (bp)	Annealing temperatures	References
Carbapenemase genes				
blaKPC – F **blaKPC - R**	ATGTCACTGTATCGCCGTCT TAGACGGCCAACACAATAGG	785	56	Ogbolu. and Webber. 2014
blaIMP-F **blaIMP-R**	CAT GGT TTG GTG GTT CTT GT ATA ATT TGG CGG ACT TTG GC	488	55	Elmonir *et al*., 2021
blaNDM-F **blaNDM-R**	GGTTTGGCGATCTGGTTTTC CGGAATGGCTCATCACGATC	621	56	Nordmann *et al*., 2011
blaVIM-F **blaVIM-R**	GTTTGGTCGCATATCGCAAC AATGCGCAGCACCAGGATAG	382	56	Mohammed.*et al*.,2015
blaOXA-48-F **blaOXA-48-R**	TTCGGCCACGGAGCAAATCAG GATGTGGGCATATCCATATTCATCGCA	240	56	Ogbolu. and Webber. 2014

Table 2: Antibiotic resistant pattern of presumptive identified Enterobacteriaceae from poultry wastes.

	Antibiotics N (%R)							
Presumptive isolates (Total number)	**ATM**	**TGC**	**AZM**	**FEP**	**CAZ**	**STX**	**DOR**	**CT**
Klebsiella aereogenes(3)	2 (66.7)	1(33.3)	2(66.7)	3 (100)	3 (100)	3 (100)	3 (100)	2(66.7)
Dickeya Chrysanthemi(1)	1 (100)	1(100)	0 (0)	1 (100)	1(100)	1 (100)	1 (100)	0(0)
Trabulsiella guamensis(2)	2 (100)	1 (50)	2(100)	2 (100)	1(50)	2 (100)	2 (100)	1 (50)
Kluyveraascorbata(13)	13(100)	9(69.2)	10(76.9)	12(92.3)	12(92.3)	13(100)	13(100)	7(53.8)
Serratia liquefaciens(1)	1 (100)	0 (0)	1(100)	1 (100)	1 (100)	1(100)	1 (100)	0(0)
Raoultell (Klebsiella) planticola(3)	3 (100)	1(33.3)	1(33.3)	3 (100)	3 (100)	3(100)	3 (100)	1(33.3)
Cedecealapagei(1)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	1(100)	1 (100)	1(100)
Salmonella spp. *(diarizonae)* (2)	2 (100)	1 (50)	2 (100)	2 (100)	1(50)	2(100)	2 (100)	0 (0)
Serratia nematodipila (3)	2 (66.7)	2(66.7)	2 (66.7)	3 (100)	2 (66.7)	2(66.7)	3 (100)	2(66.7)
Enterobacter cloacae (1)	1 (100)	1(100)	1(100)	1 (100)	1 (100)	1(100)	1 (100)	1(100)
Citrobacter freundiu(1)	1 (100)	0 (0)	0 (0)	1 (100)	1(100)	1(100)	1 (100)	0(0)
Proteus vulgaris (2)	1 (50)	0 (0)	0 (0)	1 (50)	1(50)	2(100)	2 (100)	1(66.7)
Klebsiella oxytoca(5)	4 (80)	2 (40)	2 (40)	5 (100)	4(80)	4 (80)	5 (100)	2(40)
Providencia rustigianii(1)	0 (0)	1(100)	0(0)	0 (0)	0(0)	1(100)	1 (100)	0(0)
Citrobacter farmeri(2)	1 (50)	1 (50)	1 (50)	2 (100)	2(100)	2(100)	2 (100)	0(0)
Citrobacter koseri(4)	2 (50)	1 (25)	1 (25)	4 (100)	3 (75)	2 (50)	4 (100)	2(50)
Edwardsiellatarda(1)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1(100)	1 (100)	0 (0)

Note: R (Resistance), ATM (aztreonam), TGC (tigecycline), AZM (azithromycin), FEP (cefepime), CZA (ceftazidime), SXT (sulfamethoxazole), DOR (doripenem), CT (colistin).

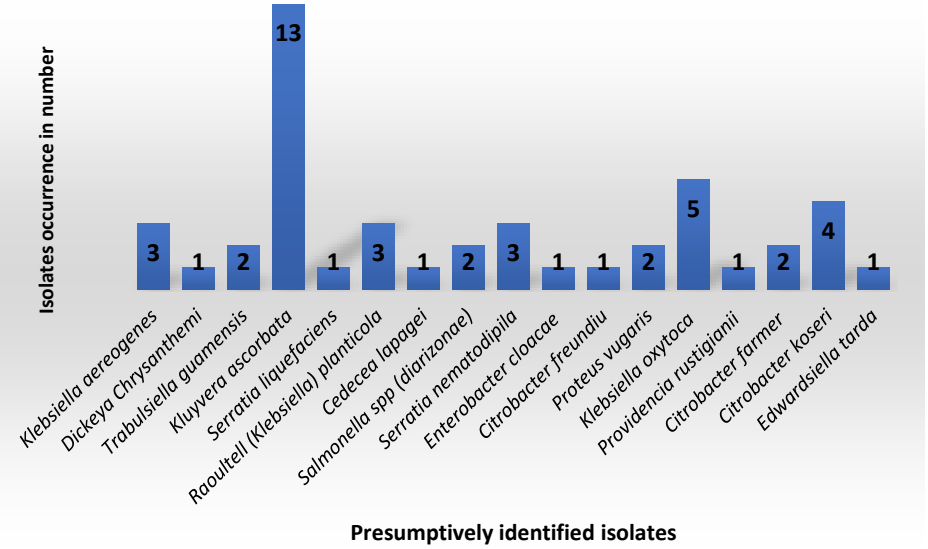

Figure 1: Frequency of occurrence of Enterobacteriaceae recovered from poultry litters.

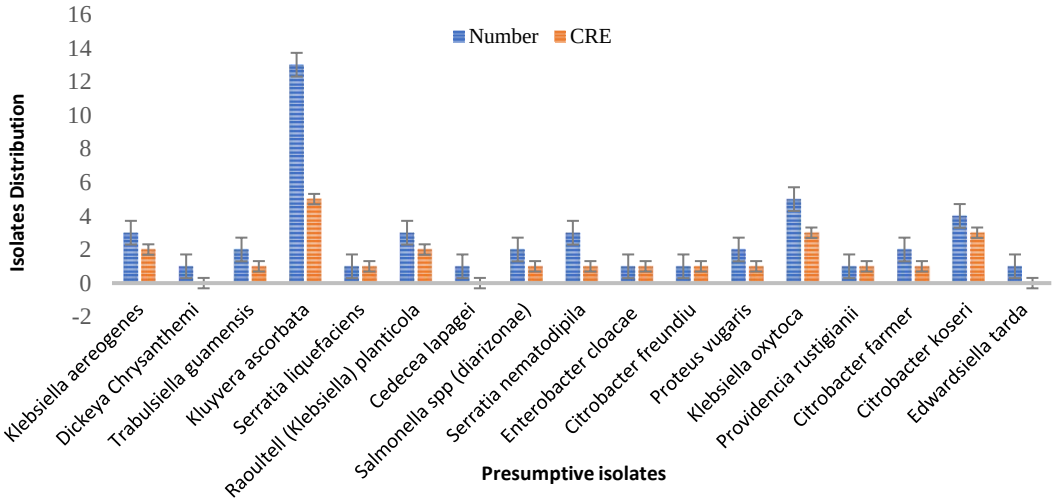

Figure 2: Distribution of Enterobacteriaceae isolates recovered from poultry wastes and their corresponding carbapenem resistant (CR) values.

100% of the phenotypically characterized carbapenem resistant isolates of *K. aereogenes, T. guamensis, S. liquefaciens, R. planticola, Salmonella spp., S. nematodipila, E. cloacae, P. vulgaris, K. oxytoca, P. rustigianii, C. farmeri* and *C.koseri* were all positive for blaIMP gene, while four out of five (80%), namely: K. ascorbata were positive for blaIMP gene. Only one out of the two isolated *Trabulsiella guamensis* positive to *blaKPC gene. C. koseri, R. planticola, S. nematodipila,C. freundiiand C. koseri* were positive to blaOXA-48 gene.

Carbapenem-resistant Enterobacteriaceae is one of the serious emerging health issues which can lead to prolonged carbapenem-related infections. Numerous enteric bacterial organisms have been reported to be detected in poultry litter samples. The organisms were identified on the basis of their morphology, Gram staining, biochemical test, carbapenemase screening, antibiotics susceptibility tests and molecular carbapenemase genes detection. High rate of resistance was detected amongst the isolates documented in this study which is an indication that the poultry environments are potential health risk environments harboring CRE organisms due to the fact that 100% of the isolates expressed multiple antibiotic resistance to commonly used antibiotics. This observation is however similar to the study conducted by (23). ESβL producing *Klebsiella* spp. isolated from poultry samples in Owerri,

Nigeria, were also documented to be completely resistant to ampicillin, ceftazidime, cefotaxime, which correlates to the results of the present study. The reports of (24) in China claimed high resistance of 98% amongst Enterobacteriaceae from poultry litters. Contrary to (25), who detected no resistance to imipenem and meropenem. Enterobactericeae isolated from river and aquaculture water samples expressed high resistant rates to ampicillin, cefotaxime, and imipenem (99%, 83% and 77%) from river water samples with 95% and 86% for ampicillin and ceftazidime from aquaculture water samples (26).

The presence of antimicrobial resistant bacteria in livestock wastes in the environment causes for an alarm, not only because of the promiscuous nature of bacteria to transfer from one environment to another, but also due to the ability of transferring resistance markers from one bacterium to another, even to humans, especially after prolonged contact with other bacteria in the same environment. Indeed, the livestock environment serves as a perfect habitat for the transfer and dissemination of resistant pathogenic bacteria. These multi-drug resistant organisms can also acquire additional resistance markers (27). The varied resistance patterns identified amongst the livestock isolates indicate a great level of variability among them which indicate the complex nature of the antimicrobial response to microorganisms. However, this could be as a result of the different and varied types of antimicrobials applied on farms for both prophylaxis and therapeutics which confer a selection pressure on the organisms. With such resistance level, it can be inferred that microbial activities on poultry farms are high, and resistance markers and microbes can be transferred from one location to the other. In this study, 92% CRE were recorded for blaIMP gene while only *Trabulsiella guamensis* was positive for blaKPC gene, 16.6% of the CRE harboured blaOXA-48 gene in comparison with other studies on poultry animals and their environment. Previous research reported that none of the β-lactamase and carbapenemase genes were detected in the carbapenem-resistant *Acinetobacter* isolates (28). In addition, Enterobacteriaceae found in different fecal and environmental samples on pig farms harboredblaIMP-27 as reported by an earlier study (29).

According to the reports of (30) who reported K. pneumoniae with blaOXA−48, blaKPC and blaNDM genes recovered from broiler-poultry farming litters in Egypt.The study of (31) also reported 245 CRE isolates from poultry farms and their slaughterhouses, dogs, sewage, wild birds, flies, and farmers in China. The authors identified blaNDM in 21.8% of the E. coli isolates, 7.4% on *K. pneumoniae* while 3.9% in E. cloacae, with respect to different variants of blaNDM−5, blaNDM−9, blaNDM−1, and blaNDM−7. P. mirabilis with blaNDM, blaOXA−1, blaOXA−10 genes were isolated from chicken in their slaughter houses and chicken farms according to the study of (32). In addition, (33) also reported blaNDM-1 in isolates *P. mirabilis* in broiler chickens.

Recommendation

Poultry farm owners must ensure that poultry farms are located far away from residential places, to prevent transference of diseases caused by resistant microorganisms. In addition, continuous research on one health should be encouraged with strict avoidance of carbapenems in animal husbandry.

CONCLUSION

There is an urgent need to carefully monitor antimicrobial resistance in food-producing animals and foods to avert the outbreak of CRE into the community. Satisfactory teamwork amidst veterinary medicine and human medicine, joined with consistent monitoring and prudent use of antimicrobials in farm foods is very necessary in order to consider whether consumers may be exposed to CRE or not. More so, exclusion of the use of carbapenems in food producing animals, upgrading of sanitary in biosecurity, hygienic conditions of animal husbandry and the application of different measures to antimicrobials would lessen the need on the usage of antimicrobials and the increase of resistant bacteria in food-producing animals.

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