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OCCURRENCE AND ANTIBIOGRAM OF GENERIC EXTENDED-SPECTRUM CEPHALOSPORIN-RESISTANT AND EXTENDED-SPECTRUM β-LACTAMASE-PRODUCING ENTEROBACTERIA IN HORSES

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ABSTRACT

This study was conducted to isolate generic extended-spectrum cephalosporin (ESC)-resistant and extended-spectrum β-lactamase (ESBL)-producing enterobacteria from horses in Nigeria, and to determine the antibacterial resistance profile. Rectal swabs were collected from 155, systematic randomly selected, apparently-healthy horses. Isolation of ESC-resistant enterobacteria was done using Mac Conkey agar with ceftazidime. ESBL production was assessed by combination disc method. Resistance of the isolates was determined using disc diffusion method. Out of 155 samples, 5.2% gave positive growth. From these, 11 ESC-resistant enterobacteria comprising of 36.4% *E. coli*, 36.4% *Salmonella* spp. and 27.2% *Proteus* spp., were obtained. From 11 isolates, 45.5% consisting of all the 4 *E. coli* and 1 *Proteus* isolates, were ESBL-producers, these were recovered from 4 (2.6%) out of the 155 horses sampled. Resistance of the *E. coli* isolates was 25% to aztreonam (AZT), 75% to amoxicillin-clavulanic acid (AMC), gentamicin (GEN), perfloxacin (PEF), and sulphamethoxazole-trimethoprim (SXT-TRI), 50% to ofloxacin (OFL) and 100% to ampicillin (AMP), ceftazidime (CTZ), cefotaxime (CTX), chloramphenicol (CHL), streptomycin (STR), tetracycline (TET), sparfloxacin (SPA), ciprofloxacin (CIP), norfloxacin (NOR) and enrofloxacin (ENR). Resistance of the *Salmonella* isolates was 50% to PEF and 100% to CTZ, CTX, AMP, AZT, AMC, CHL, GEN, STR, TET, SPA, CIP, OFL, NOR and ENR. Resistance of the *Proteus* isolates was 25% to AMC, CHL, STR, TET, SPA and NOR, and 100% to CTZ, CTX, AZT and AMP. Resistance of the isolates to more than 3 classes of antibacterial agents tested was 75% for *Proteus* and 100% for *E. coli* and *Salmonella*, respectively. This study showed that horses in Nigeria are potential reservoirs and disseminators of ESC-resistant and ESBL-producing *Enterobacteriaceae.*

Key words: antibiogram, extended-spectrum cephalosporin-resistant, extended-spectrum β-lactamase-producing, *Enterobactericeae*, equine

INTRODUCTION

There is increased interest in extended-spectrum cephalosporin (ESC)-resistant *Enterobactericeae,* because these organisms jeopardize ESC (3rd- and 4th-generation cephalosporins which are critically-important in human and veterinary medicine) therapy and also exhibit resistance to other classes of antibacterials used in human and veterinary medicine, including chloramphenicol, fluoroquinolones, tetracycline, trimethoprim and sulphonamides (1, 2). Recently, ESC-resistant *Enterobacteriaceae* were classified as "critical priority 1 pathogens" that pose a threat to human and animal health and against which new treatment strategies are urgently needed (3), therefore there is a need to document their occurrence in different potential reservoirs. ESC resistance in *Enterobacteriaceae* is mediated by the production of β-lactamases, especially the extended-spectrum β-lactamases (ESBL) which hydrolyze all generations/subclasses of β-lactams except cephamycins and carbapenems, but are inhibited by β-lactamase-inhibitors (e.g., clavulanic acid), and Ampicillinase (Amp) C β-lactamases which also hydrolyze β-lactamase-inhibitors (4).

Nigeria has an estimated horse population of 200,000-240,000 (5, 6); most of these horses are

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raised in the northern region of the country where they are used for pleasure riding, sports (especially polo games), ceremonies (such as dubar festival), entertainment, security purposes, crowd control (in police mountain troops), research and livestock rearing (together with ruminants) in a sedentary system where they are used for transport activities in the farm (7, 8). In the southern part of Nigeria, horses are mainly used for polo games (9). Due to intensive management and extensive uses of these horses and their vivacious temperament, they are often prone to diseases and physical injuries (10, 11) which are often managed with antimicrobials by veterinarians or non-professionals without veterinary supervision. There are no regulations guiding the use of antimicrobials in Nigeria (12). Because horses are prized animals, critically-important antibiotics, including extended-spectrum cephalosporins (ESCs) may be used in treating horses in Nigeria in order to ensure therapeutic success. Thus, these horses may harbour ESC-resistant/ESBL-producing enterobacteria. These organisms harboured by these horses could be discharged into the environment, thus serving as reservoirs and disseminators of genes encoding β-lactam resistance and ESBL (13). These genes present in the discharged enterobacteria could be acquired by horizontal transfer to pathogenic human bacteria, thereby complicating infection and compromising antibacterial therapy (13). Direct physical contact between these horses and humans especially their owners, handlers/groomers, jockeys, children and veterinarians, is a putative risk for transmission of ESC-resistant/ESBL-producing enterobacteria (14, 15). Indirect contact via contaminated formites (such as grooming gears and saddles), environment and food chain contaminated from environmental discharges, are also a potential risk for transmission of these organisms (16). Evidence supports zoonotic transmission of ESC-resistant/ESBL-producing enterobacteria from horses (2, 17, 18). Spent (used) horses are transported from the northern region to the southern part of Nigeria where they are slaughtered as source of animal protein. The presence of ESC-resistant/ESBL-producing enterobacteria in the faeces of these horses at slaughter represents a risk for carcass contamination and subsequent contamination of retail meat products, because inadequate hygienic practices are employed during meat processing in Nigeria (13, 19). A close association between livestock, horses and humans is inevitable in northern Nigeria (8), which could result in the exchange of ESBL-producing enterobacteria between these hosts. This will consequently result in the fast spread of these organisms in the human and animal population in the country, since majority of the livestock in Nigeria are raised in the northern region. Thus, there is need to screen horses in Nigeria as potential reservoirs of ESBL-producing enterobacteria. Moreover, determination of phenotypic antibacterial resistance/susceptibility profile of ESC-resistant organisms is important for empiric treatment of infections associated with these organisms in horses (20).

The One Health-oriented approach of antimicrobial resistance surveillance involves monitoring of the spread of antibacterial resistance among non-clinical isolates of commensal organisms in hobby and food-producing animals (21). There are a number of reports on isolation of ESC-resistant/ESBL-producing enterobacteria from clinical samples of horses (2, 17, 18, 22-26), but surveillance studies assessing healthy horses as potential reservoirs and disseminators of ESC-resistant/ESBL-producing enterobacteria are rather scanty and included reports in the United Kingdom (27, 28) and Czech Republic (17). In Nigeria, investigators have screened animals such as chickens (29-32), ruminants (33-35), pigs (13, 34) and dogs (36) as potential reservoirs of ESC-resistant/ESBL-producing enterobacteria. No study has been conducted to screen horses in Nigeria as potential reservoirs of ESC-resistant/ESBL-producing enterobacteria. The objective of this study, therefore, was to determine the occurrence of ESC-resistant and ESBL-producing enterobacteria in horses at slaughter in the Obollo Afor market, Udenu Local Government Area (L. G. A.), Enugu State Southeast Nigeria and determine the antibacterial resistance/susceptibility profile of the isolates.

MATERIAL AND METHODS

Study area

Obollo-Afor is a town in Udenu L. G. A. of Enugu State, Southeastern Nigeria. It is geographically located at coordinates 6.9153° N and 7.5139° E. Obollo-Afor market is a major horse selling and slaughtering point (about 25 horses are killed every 4 days) in Enugu State.

Sampling

Horses presented for slaughter at the Obollo-Afor market/slaughter slab between February

and June, 2017 were sampled. These ready-to-slaughter horses were apparently-healthy and they have been used for various purposes in the northern part of Nigeria and transported to the study site for slaughter and consumption. One hundred and fifty-five horses, consisting 30% of total slaughter within the period of the study, were selected using a 1:5 systematic random sampling technique. Prior to slaughter, non-duplicate rectal swabs were collected from each of the horse using a sterile swab stick. The samples were transported aseptically in ice packs and processed within 1 hour of collection in the Veterinary Microbiology Laboratory, Department of Veterinary Pathology and Microbiology, University of Nigeria, Nsukka.

Isolation and generic identification of ESC-resistant enterobacteria from horses

The swabs were cultured on Mac Conkey agar amended with 4μg/mL of ceftazidime and incubated at 37°C for 24 hours. The morphology of different colonial types were described and recorded appropriately. Purification of the isolates was done by sub-culturing on plain Mac Conkey agar and incubated at 37°C for 24 hours. Pure cultures of the isolates were then inoculated onto nutrient agar slants, incubated at 37°C for 24 hours and stored in refrigerator at 4°C as stock cultures until needed for further analysis. Phenotypic characterization of the isolates to generic level was done by subjecting them to various tests such as Gram staining, oxidase, catalase, urease, methyl red, citrate and triple sugar iron agar test, and sub-culturing on eosin methylene blue agar following standard methods per Cheesbrough (37).

Determination of antibiogram of generic ESC-resistant enterobacterial isolates from horses

Antibacterial resistance/susceptibility profiles of the generic ESC-resistant and ESBL-producing enterobacterial isolates were determined by the disc diffusion method (38), using 17 antibacterial agents in 7 classes: fluoroquinolones – ciprofloxacin (CIP) (5 μg), perfloxacin (PEF) (5 μg), ofloxacin (OFL) (5 μg), sparfloxacin (SPA) (5 μg), norfloxacin (NOR) (10 μg) and enrofloxacin (ENR) (5 μg), folate pathway inhibitors - sulphamethoxazole-trimethorpim (SXT-TRI) (25 μg), β-lactams/ cephalosporins – ampicillin (AMP) (10 μg), aztreonam (AZT) (30 μg), ceftazidime (CTZ) (30 μg), cefotaxime (CTX) (30 μg), meropenem (MPN) (10 μg), β-lactam-β-lactamase inhibitors - amoxicillin-clavulanic acid (AMC) (20/10 μg), tetracycline (TET) (30 μg), aminoglycosides – gentamicin (GEN) (10 μg) and streptomycin (STR) (10 μg), and phenicol – chloramphenicol (CHL) (30 μg). Results of the antibacterial resistance/ susceptibility testing were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) (39) guidelines for aerobic pathogens. Intermediately-susceptible isolates were classified as resistant in this study.

Detection of extended-spectrum β-lactamase production by generic ESC-resistant enterobacteria from horses

The isolates were screened for ESBL production using the combination disc method (cefpodoxime/ clavulanic acid [10:1μg] and cefpodoxime alone [10μg] on Mueller-Hinton agar. Each test isolate that produced an inhibition zone with a diameter difference of 5mm or more between the combination disc and cefpodoxime alone was considered as an ESBL-producer (38, 39).

Data analysis

The frequencies of occurrence of the enterobacterial genera and resistance of the isolates to antibacterial agents were entered into Microsoft Excel version 2010 (Microsoft Corporation, Redmond, USA) and their percentages calculated.

RESULTS

Occurrence of generic ESC-resistant enterobacteria in horses

Out of 155 rectal swabs cultured, 8 (5.2%) gave positive culture of ESC-resistant enterobacteria. From these, 11 enterobacterial isolates consisting of 4 (36.4%) *Escherichia coli*, 4 (36.4%) *Salmonella* spp. and 3 (27.2%) *Proteus* spp., were obtained.

Occurrence of generic ESBL-producing enterobacteria in horses

Out of the 11 ESC-resistant isolates, 5 (45.5%) comprising of all the 4 (80%) *E. coli* isolates and 1 (20%) *Proteus* isolate, were ESBL-producers. Three of the ESBL-producing *E. coli* were from 3 horses, while 1 ESBL-producing *E. coli* and the ESBL-producing *Proteus* isolates were obtained from 1 horse, thus 4 (2.6%) out of the 155 sampled horses harboured ESBL-producing enterobacteria.

Antibiogram of generic ESC-resistant and ESBL-producing enterobacterial isolates from horses

Out of the 4 ESBL-producing *E. coli* isolates, all were resistant to CTZ, CTX, AMP, STR, TET, CHL, SPA, CIP, NOR and ENR, 3 (75%) to AMC, GEN, PEF and SXT-TRI, 2 (50%) to OFL and 1 (25%) to AZT (Fig. 1). All the *E. coli* isolates were susceptible to MPN.

Out of the 4 ESC-resistant *Salmonella* isolates, all were resistant to CTZ, CTX, AMP, AZT, AMC, GEN, STR, CHL, TET, SPA, CIP, OFL, NOR and ENR and 2 (50%) to PEF (Fig. 2). All the *Salmonella* isolates were susceptible to MPN and SXT-TRI.

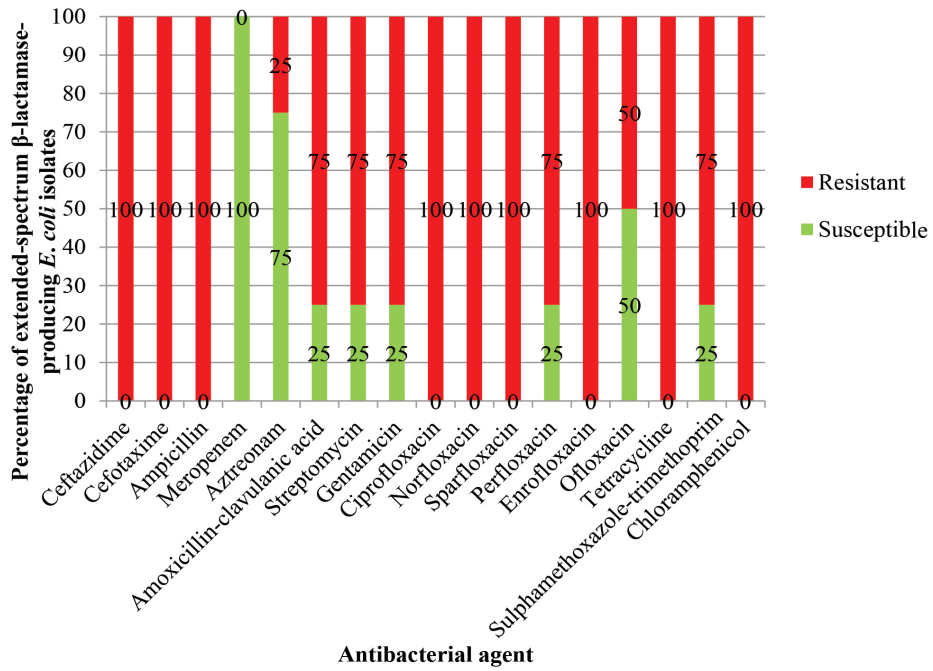

Figure 1. Antibiogram of 4 generic extended-spectrum β-lactamase-producing *Escherichia coli* isolates from horses

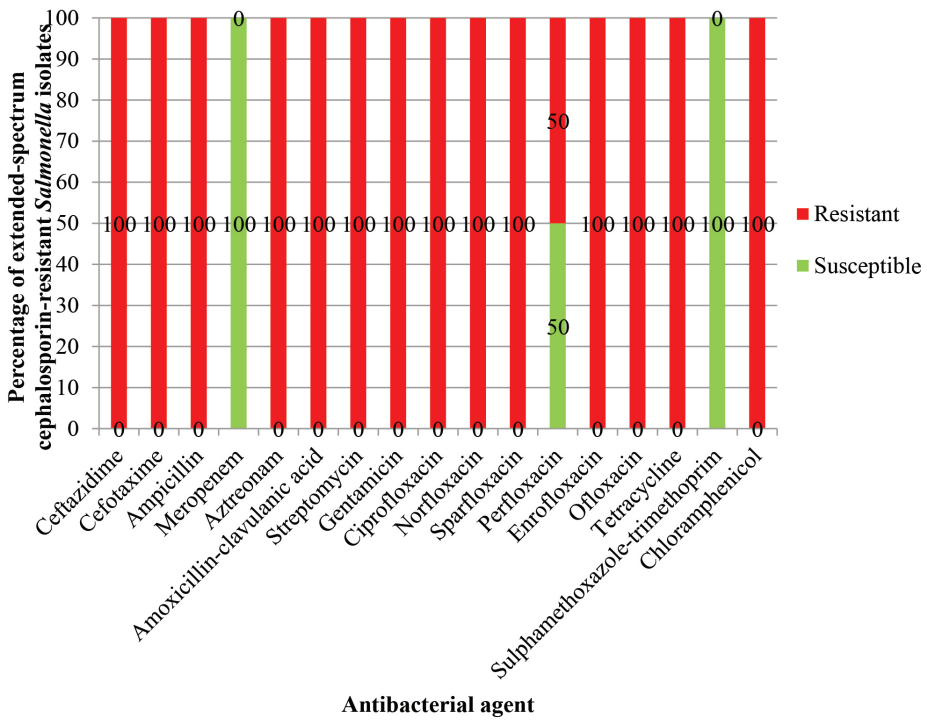

Figure 2. Antibiogram of 4 generic extended-spectrum cephalosporin-resistant *Salmonella* isolates from horses

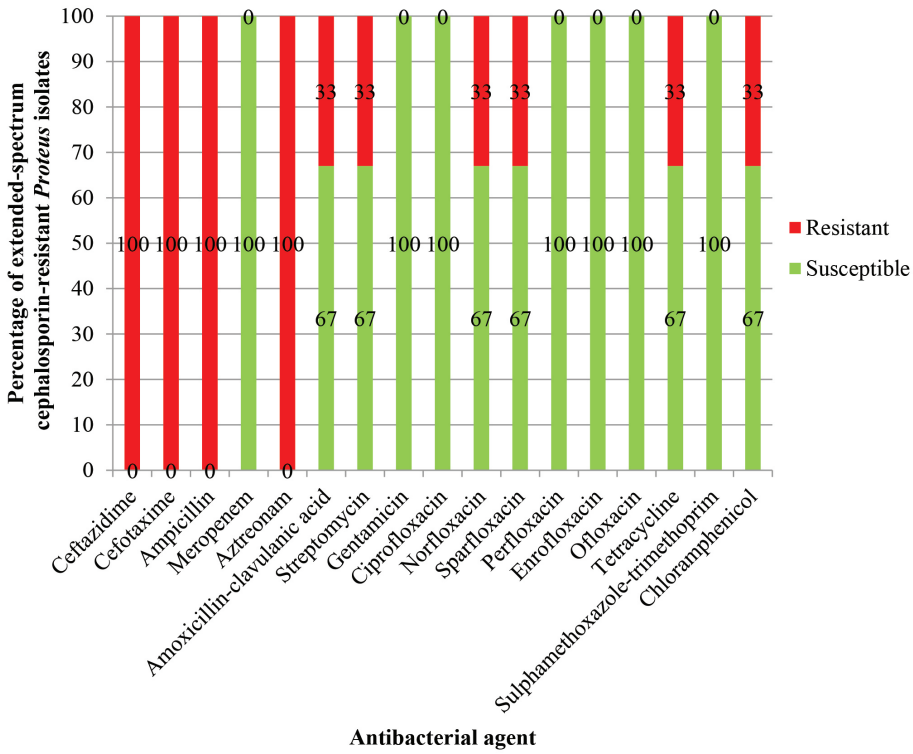

Figure 3. Antibiogram of 3 generic extended-spectrum cephalosporin-resistant *Proteus* isolates from horses

Out of the 3 ESC-resistant *Proteus* isolates, all were resistant to CTZ, CTX, AZT and AMP, 33% to AMC, CHL, STR, TET, SPA and NOR (Fig. 3). All the *Proteus* isolates were susceptible to MPN, SXT-TRI, GEN, PEF, CIP, OFL and ENR.

All the *E. coli* and *Salmonella* isolates were resistant to 3 or more classes of antibacterial agents tested. Two ESC-resistant *Proteus* isolates were resistant to 2 classes of antibacterial agents tested, while the ESBL-producing isolate exhibited resistance to 3 or more classes of antibacterial agents tested.

DISCUSSION

The fact that 8 (5.2%) of the 155 non-duplicate rectal swab samples cultured using Mac Conkey agar supplemented with 4µg/mL of ceftazidime (an oxyimino-cephalosporin) gave positive growth, suggested that a sizeable percentage of horses in the study area are colonized by ESC-resistant enterobacteria. Isolation of 11 ESC-resistant enterobacteria in 3 genera (4 *E. coli*, 4 *Salmonella* and 3 *Proteus* isolates) from the 8 horses among 155 apparently-healthy horses in this study, suggested that the isolates produced ESBL and/or AmpC β-lactamases (15, 23). This finding calls for concern because these horses potentially serve as reservoirs and disseminators (by faecal shedding) of these organisms into the environment, thereby posing a health threat to individuals that get in contact with them (2, 20, 22). Veterinarians, jockeys, caretakers and owners of these horses could acquire these organisms following contact with faeces from the horses or contact with formites contaminated by the organisms (2, 14, 15, 40). Since the horses are meant for slaughter, these organisms could also be acquired by the butchers, slaughterhouse workers and consumers following contact with and/or consumption of meat and associated products from the horses (14, 20). These individuals could serve as source of dissemination of these organisms to the public (14, 20). Possible sources of these organisms in the sampled horses include nosocomial transmission from previous hospital visits, contaminated environment and formites, as well as contaminated feed and/or drinking water (2, 20, 41). The use of β-lactams, especially the extended-spectrum cephalosporins in horses, has also been reported to induce selection for ESC resistance (4, 20). Although the medical history of the sampled horses in this study could not be traced since they were used in the northern region and transported

to the study site, it is possible that they might have been treated earlier with a β-lactam antibiotic. It is equally possible that the horses might also have been treated earlier with a β-lactam antibiotic at the study site.

The 5.2% ESC-resistant enterobacteria occurrence in this study is similar to 5.7% ESC-resistant *E. coli* occurrence among 296 faecal *E. coli* isolates from randomly selected hospitalized horses and horses in livery premises reported by Ahmed et al. (42) in Northwest England. It is worth to note that in Enugu State southeastern Nigeria, Ugwu et al. (13) reported 24.2% faecal ESC-resistant enterobacterial occurrence among 190 randomly selected healthy pigs using 2 µg/mL of cefotaxime-amended MCA, while Anyanwu et al. (36) used 4 µg/mL of ceftazidime-amended MCA (similar to the current work) and recorded 27% faecal ESC-resistant enterobacteria occurrence among 100 randomly selected healthy dogs in Nsukka Enugu State Nigeria, respectively. These results are higher than the findings (5.2%) of this study. The variation in the results of these studies may be related to the differences in the method of sampling, isolation/processing, type and concentration of ESC used in supplementing the media for primary isolation, concentration of ESC used in the sensitivity test, method used in determining ESC resistance, and usage of β-lactams in the various study areas (20, 36). The concentration of ESC used in this study has also been used for isolation of ESC-resistant *Enterobacteriaeae* elsewhere (43).

The lower ESC-resistant enterobacterial occurrence in this study, suggested a lower ESC selection pressure among enterobacteria colonizing these horses than in those that colonized dogs and pigs in Nigeria (13, 36). Low ESC selection pressure in enterobacteria in this study may be as a result of infrequent β-lactam antibiotic treatment given to these horses raised in the northern region of Nigeria, where they are used for several purposes, and then transported down to the southern region of the country where they are kept for a short time and slaughtered for food (10, 11). It is also possible that a higher occurrence rate of ESC-resistant enterobacteria than as observed in this study might have been recorded, if simple random sampling was used rather than systematic random sampling.

In this study, 34.6% ESC-resistant *E. coli* and *Salmonella* occurrence and 27.2% ESC-resistant *Proteus* occurrence was observed. Anyanwu et al. (36) reported *E. coli* as the predominant (40.7%) ESC-resistant enterobacteria in dogs, whereas Ugwu et al. (13) recorded *Klebsiella* as the most prevalent (67.4%) ESC-resistant enterobacterial isolates from pigs. The differences in the predominance of enterobacteria genera isolated in these studies may be related to the disparity in usage of ESCs in management of the different animal species.

Detection of ESBL-producing enterobacteria in 4 (2.6%) of the 155 sampled horses in this study, further indicated that the horses are potential reservoirs of ESBL-producing enterobacteria, while it also further proves that the isolates produced ESBL. The result also suggested a low occurrence of ESBL-producing enterobacteria in horses in the study area. It may also mean that a low proportion of enterobacteria colonizing horses in the study area have acquired genes encoding ESBL. The 2.6% ESBL-producing enterobacteria occurrence in this study is lower than 6.3, 84, 32 and 100% ESBL-producing *E. coli* occurrence in faecal samples of 650 horses in a community in UK (28), faecal/wound samples of 91 hospitalized and hospital resident horses in the Netherlands (2), faecal samples of 37 horses in equine clinic and horse riding back centers in Czech Republic (17) and faecal samples of 10 horses that received antibacterial treatment in Denmark (44), respectively. Differences in these studies could be attributed to variations in health status of the animals (whether sick or healthy), type of sample analyzed, method of detection of ESBL production (whether phenotypic or molecular), and degree of contamination of horses'/animals' environment and colonization (2, 36, 44). In the experiment presented hereby, combination disc diffusion method utilizing cefpodoxime (an ESC) with and without clavulanic acid, was employed in detecting ESBL production (38, 39). This recommended method has proved to be reliable (but not as molecular methods) in detection of ESBL production in enterobacterial isolates (38, 39). Over-expression of non-ESBL (such as TEM-1 and OXA-1) and loss of porins could result in false ESBL phenotypes (45). Therefore, it is possible that the occurrence rate of ESBL-producing enterobacteria in horses sampled in this study may not be detected as much by the combination disc test (a phenotypic ESBL production detection method) used in this study.

Detection of 5 (45.5%) ESBL-producing enterobacteria (4/4 *E. coli* and 1/3 *Proteus* isolates) among 11 ESC-resistant enterobacteria in this study, suggested that horses in the study area are colonized by different ESBL-producing enterobacterial genera. It also suggested that either the horses already harboured the ESBL-producing organisms or that the isolates acquired genes encoding ESBL

from other enterobacteria in the gut (2). The use of antibiotics, including extended-spectrum cephalosporins, in horses and other animals are not regulated in Nigeria (12, 13). Thus, the findings also suggested that these horses might have been treated with ESCs during previous visitations to hospital or by their owners or handlers. It is also possible that they have been treated with these drugs or acquired ESBL-producing enterobacteria while awaiting slaughter at the study site. Unfortunately, there is no pre-slaughter assessment to detect the possible use of these drugs in horses in Nigeria. The finding of ESBL-producing enterobacteria in this study is worrisome, because ESBL-producing organisms rapidly transfer MDR genes (by plasmids) to other bacteria by horizontal transfer following acquisition by individuals (such as groomers, veterinarians, jockeys and owners) in direct contact with carrier animals (4). Ingestion of raw horse meat as practiced by some butchers in Nigeria and consumption of undercooked meat, are also putative risk for acquisition of ESBL-producing organisms from these horses; this is because of the high risk of carcass contamination due to poor hygienic practices employed during animal slaughter in Nigeria (13, 19). In Germany, Schmeidel et al. (22) observed 37% ESBL-producing enterobacteria occurrence among 100 ESC-resistant enterobacterial isolates from different samples of sick horses, a finding that is lesser than the result (45.5%) of this study. Vo et al. (27) detected 85.7% ESBL-production among 7 ESC-resistant enterobacterial isolates from horses in the Netherlands, this result is higher than the finding (45.5%) of this study.

Phenotypic confirmation of all the ESC-resistant *E. coli* isolates in this study as ESBL-producers is similar to the findings of Smet et al. (24) that all 5 cephalosporin-resistant *E. coli* isolates from diseased horses in Belgium were ESBL-producers. A retrospective study conducted in Switzerland, reported 60% ESBL-producing *E. coli* occurrence among 60 clinical *E. coli* isolates from different samples of horses (26), while a Dutch study observed 99.5% ESBL-producing *E. coli* occurrence among 198 faecal/wound *E. coli* isolates from horses (2); these findings are lesser than the result (100%) of the current study. Previous studies in Nigeria (which did not use ESC-amended media for primary isolation) reported 16.3-44.7% and 8.9-22.5% ESBL-producing *E. coli* occurrence among *E. coli* isolates from cattle (34, 35) and chickens (29, 31, 33), respectively. These results are also lesser than the findings (100%) of this study. Variation in the detection rate of ESBL-producing *E. coli* in these studies may be related to differences in use of ESCs in the study areas, contamination of feed, drinking water and/or environment, infection and colonization of the animals by the organisms. Detection of 4 (80%) ESBL-producing *E. coli* isolates compared against 1 (20%) *Proteus* isolate in this study, suggested that *E. coli* may be the predominant ESBL-producing enterobacterial genus colonizing horses in the study area.

Interestingly, none of the isolates in this study exhibited resistance to MPN (a carbapenem) which is the last resort drug for treating infections associated with extended-spectrum β-lactam-resistant-, ESBL- and AmpC β-lactamases-producing, and other MDR bacteria (12, 13). This finding suggested that carbapenems have not been abused in equine practice in Nigeria.

CONCLUSION

This study has shown that ESC-resistant (5.2%) and ESBL-producing (2.6%) enterobacteria are harboured by a sizeable percentage of horses slaughtered at the Obollo Afor market in Udenu L. G. A., Southeastern Nigeria. *E. coli* is the dominant genus of ESBL-producing enterobacteria colonizing horses in the study area. Thus, these horses are potential reservoirs and disseminators of ESC-resistant/ESBL-producing enterobacteria and genes encoding ESBL and AmpC β-lactamases. This has tremendous impact on the food chain, ecology and epidemiology of antibacterial resistance. Therefore, attention should be paid on the use of ESCs in horses in Nigeria. However, further studies to determine the genes encoding ESBL and/or AmpC β-lactamases in the isolates are recommended.

CONFLICT OF INTEREST STATEMENT

The authors declared that they have no potential conflict of interest with respect to the authorship and/or publication of this article.

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