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Detection and molecular characterisation of extended-spectrum β-lactamase-producing enteric bacteria from pigs and chickens in Nsukka, Nigeria☆

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ABSTRACT

Objectives: This study screened chickens and pigs slaughtered for human consumption for the presence and characteristics of extended-spectrum β-lactamase (ESBL)- and plasmid-encoded AmpC (pAmpC) β-lactamase-producing enteric bacteria.
Methods: Faecal samples from 410 broiler chickens and 100 pigs were cultured on MacConkey agar supplemented with 2 μg/mL cefotaxime. Antimicrobial resistance phenotypes of the recovered isolates were determined by disk diffusion. PCR and sequencing were performed to identify the ESBL and pAmpC gene variants and other associated resistance determinants. Genetic diversity of the isolates was analysed by phylotyping and multilocus sequence typing.
Results: ESBL-producing *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter asburiae* and *Providencia* spp. were isolated from 17 (4.1%) and 2 (2.0%) of the samples from chickens and pigs, respectively. One pAmpC-producing *E. coli* isolate was obtained from a chicken. Resistance to tetracycline, trimethoprim/sulfamethoxazole, chloramphenicol and gentamicin was exhibited by 95%, 80%, 60% and 55% of the ESBL/pAmpC-producing strains, respectively. *tet*(A) and *aac(3)-II* were the predominant genes detected in tetracycline- and aminoglycoside-resistant strains, respectively. *bla*$_{CTX-M}$, encoding CTX-M-15 (15 isolates) or CTX-M-1 variants (3 isolates), was present in all but one ESBL-producer, either alone or in combination with *bla*$_{SHV}$ and/or *bla*$_{TEM}$. The remaining ESBL-producer, a *Providencia* spp. recovered from a chicken, harboured *bla*$_{VEB}$. The only pAmpC-positive *E. coli* strain carried *bla*$_{CMY-2}$. The 11 ESBL-producing *E. coli* strains belonged to five lineages (ST226-A, ST3625-B1, ST10-A, ST46-A and ST58-B1).
Conclusions: Healthy chickens and pigs act as reservoirs of ESBL/pAmpC-producing enterobacteria that can potentially be transmitted to humans through direct contact or ingestion of contaminated meat.
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1. Introduction

Antimicrobial resistance is a growing public-health threat worldwide, and members of the Enterobacteriaceae family are among the clinically important bacteria that are rapidly developing resistance to available antibacterial agents. The most important mechanism of resistance to third-generation cephalosporins among members of the Enterobacteriaceae, particularly *Escherichia coli* and *Klebsiella pneumoniae*, is the production of extended-spectrum β-lactamase (ESBL) enzymes [1,2]. These enzymes hydrolyse the β-lactam ring of extended-spectrum β-lactam antibacterial agents (cephalosporins and monobactams). ESBL-producing bacteria pose a serious therapeutic challenge as they are frequently resistant to other classes of antimicrobial agents such as fluoroquinolones, aminoglycosides and trimethoprim/sulfamethoxazole (SXT) [3].

Three major families of ESBL enzymes have been reported, namely TEM, SHV and CTX-M types [4]. The TEM- and SHV-type ESBLs arise by point mutations leading to substitutions of key amino acid residues in the classical TEM-1/TEM-2 and SHV-1

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β-lactamases [1,2]. These ESBL types predominated in the 1980s and 1990s. However, the first non-TEM/SHV β-lactamase-producing cefotaxime-resistant *E. coli* was isolated from the faecal microbiota of a laboratory dog in Japan in 1986 [5]. The enzyme responsible for the cefotaxime resistance was designated FEC-1 and was later found to be related to CTX-M-3 type [6]. The CTX-M-type ESBLs have now emerged as the dominant types [1] and are increasingly being detected both in human and animal populations in many parts of the world [7,8]. On the basis of amino acid sequence, five groups or clusters of CTX-M-type ESBLs have been identified, namely CTX-M-1, -2, -8, -9 and -25 [2]. Healthy food animals are increasingly being reported as reservoirs of ESBL-producing enteric bacteria [7,9].

In Nigeria, most of the available reports on molecular characterisation of ESBL genes are on human isolates [10,11], with scant information on animal isolates [12,13]. The objective of this study was to screen broiler chickens and pigs slaughtered for human consumption at Ikpa market in Nsukka (Nigeria) for ESBL-producing enteric bacteria and to characterise the recovered isolates.

2. Materials and methods

2.1. Isolation and identification of extended-spectrum β-lactamase- and plasmid-encoded AmpC-producing enteric bacteria

Faecal swab samples were collected from 410 broiler chickens (210 samples between April–June 2014 and 200 samples between September–November 2015) and 100 pigs (September–November 2015) presented for slaughter at Ikpa market/slaughterhouse. Samples were collected once a week during the sampling period, and not more than 20 chickens and 10 pigs were sampled at each visit; each sampled animal came from a different farm or owner. Swab samples were obtained from the cloaca and rectum of chickens and pigs, respectively, just before slaughter. Each swab sample was placed into a sterile tube, was transported to the laboratory and was processed within 2 h of collection. Samples were inoculated on MacConkey agar supplemented with 2 μg/mL cefotaxime (CMCA) plates and inoculated plates were incubated at 37 °C for 18–24 h. One representative colony of each morphological type from the CMCA plate was picked and was subcultured on MacConkey agar to obtain a pure culture. The pure cultures colonies were screened for ESBL production using the combination disk method [cefpodoxime/clavulanic acid (10:1 μg) and cefpodoxime alone (10 μg)] on Mueller–Hinton agar. Each test isolate producing an inhibition zone diameter difference of ≥5 mm between the combination disk and the cefpodoxime disk was considered an ESBL-producer [14]. Each isolate producing a zone difference of <5 mm was screened for susceptibility to cefoxitin (30 μg) by the disk diffusion method. Cefoxitin-resistant isolates were considered as presumptive AmpC-producers. The ESBL- and presumptive AmpC-producers were subcultured on brain–heart infusion agar, were incubated overnight at 37 °C and were processed for species identification using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) [15] and 16S rRNA sequencing. Bacterial genomic DNA was extracted using an InstaGene™ Matrix Kit (Bio-Rad, Hercules, CA) and the PCR assay was performed using previously described primers (uni16-F, AGAGTTTGATYMTGGCTCAG; and uni16-R, GGYTACCTTGTTACGACTT) [16,17]. For further molecular analysis, we only focused on bacteria producing acquired β-lactamases, i.e. ESBLs and plasmid-encoded AmpC (pAmpC) β-lactamases. Chromosomally-encoded AmpC β-lactamases were excluded.

2.2. Antimicrobial susceptibility testing of extended-spectrum β-lactamase- and plasmid-encoded AmpC-producing enteric bacteria

Antimicrobial susceptibility profiles of the bacterial isolates were determined by the disk diffusion method using 13 antimicrobial agents: ampicillin (10 μg); amoxicillin/clavulanic acid (30 μg); cefoxitin (30 μg); ceftazidime (30 μg); cefotaxime (30 μg); imipenem (10 μg); nalidixic acid (30 μg); ciprofloxacin (5 μg); chloramphenicol (10 μg); SXT (25 μg); gentamicin (10 μg); tobramycin (10 μg); and tetracycline (30 μg). The results of antimicrobial susceptibility testing were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) for veterinary pathogens [18].

2.3. Detection of extended-spectrum β-lactamase- and plasmid AmpC-encoding genes and other antimicrobial resistance genes

ESBL-producing bacteria were screened for the presence of genes encoding TEM-, SHV-, OXA-1- and CTX-M-type β-lactamases by PCR using specific primers and conditions reported previously [9]. Detection of acquired *ampC* genes among presumptive AmpC-producing isolates was done using a multiplex PCR assay [19]. Absence of the classic ESBL genes in an isolate prompted the search for minor ESBL families such as VEB [20]. The amplicons obtained were sequenced and the sequences were compared with those in the GenBank and Lahey Clinic (http://www.lahey.org/Studies/) public databases to identify β-lactamase variants. Tetracycline- and gentamicin-resistant isolates were screened by PCR for the presence of *tet*(A), *tet*(B), *tet*(C) and *tet*(D) genes and *aac(3)-II* genes, respectively [21].

2.4. Multilocus sequence typing (MLST) and phylotyping of extended-spectrum β-lactamase- and plasmid-encoded AmpC-producing Escherichia coli strains

To identify the genetic lineages of the ESBL- and pAmpC-producing *E. coli* strains, DNA sequencing of the internal fragments of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) was performed. The allelic profile of each strain was determined and the sequence type (ST) was assigned in accordance with the *E. coli* MSLT website (http://mlst.ucc.ie/mlst/dbs/Ecoli). The *E. coli* strains were classified into one of the seven major phylogenetic groups (A, B1, B2, C, D, E and F) using the quadruplex PCR method proposed by Clermont et al. [22].

3. Results and discussion

3.1. Extended-spectrum β-lactamase- and plasmid-encoded AmpC-producing enteric bacterial species isolated

Samples from 18 (4.4%) of the 410 broiler chickens and 2 (2.0%) of the 100 pigs yielded growth on CMCA plates. Of the 20 isolates recovered on CMCA, 19 were phenotypically confirmed as ESBL-producers by the combination disk method using cefpodoxime/clavulanic acid and cefpodoxime disks. The remaining isolate was resistant to cefoxitin and was therefore a presumptive AmpC-producer. The isolates recovered on CMCA were identified into four species, namely *E. coli* (11 strains), *K. pneumoniae* (7 strains), *Enterobacter asburiae* (1 strain) and *Providencia* spp. (1 strain) (Table 1). Ten of the *E. coli*, all seven *K. pneumoniae* and the single *E. asburiae* and *Providencia* spp. strains were ESBL-producers, whilst one *E. coli* strain was a pAmpC-producer. Among the genera of the family Enterobacteriaceae, ESBL production is predominantly found in *E. coli* and *Klebsiella* spp. [2].

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