Low Incidence of Extended Spectrum Beta Lactamase (ESBL) Detection From Enterobacteriaceae Isolated From Pig Faeces.

Itaman, V.O[1] * Nwankwo, E.O,' Erhunmwunse, A.S[2]. andAdeleye, LA[3].

1 Department of Microbiology, Michael Okpara University of Agriculture, Umudike, Abia, Nigeria.

2Department of Soil Science, Obafemi Awolowo University, lle-Ife, Nigeria.

3Department of Microbiology, University of Lagos, Lagos, Nigeria.

Corresponding author: Email: Tel: 08067559876.

Abstract: This study assessed the low incidence of ESBL detect and associated antimicrobial/antibiotic resistance from *Enterobacteriaceae* isolated from pigs. A total of twenty-six (26) isolates from the forty-five (45) faecal piggery samples studied were Gram negative bacteria which were further characterized using standard biochemical procedures as : *Citrobacter freundii* 7(26.9%), *E. coli* 5(19.2%), *Klebsiella pneumonia* 5(19.2%), *Enterobacter aerogenes* 2(7.7%), *Salmonella* spp. 6(23.1%), and *Shigella* spp. 1(3.9%). Antimicrobial susceptibility of these isolates to the following antibiotics: gentamicin (GEN) lOug, cefuroxime (CRX) 30 ug, ofloxacin (OFL) 5 ug, ceftazidime (CAZ) 30 ug, augmentin (AUG) 30 ug, cefixime (CXM) 5 ug, nitrofurantin (NIT) 30 ug, ciprofloxacin (CPR) 5 ug was determined using the Kirby-Bauer disc diffusion method; twelve (12) of the isolates showed resistance to two or more of the antibiotics. The major finding is the presence of multi-drug resistant *E.coli* and *Klebsiella pneumonia* to commonly used antibiotics such as augmentin, ceftazidime, cefixime, and cefuroxime. These multi drug resistant isolates were screened for possible ESBLs production using the double disc synergy test (DDST) and none was ESBL positive. Pig faeces do not pose a high risk of contamination with ESBL producing bacteria of the *Enterobacteriaceae* group in the environment.

Keywords: ESBL, Enterobacteriaceae, pig faeces

Introduction

Antimicrobial agents in veterinary medicine are used to treat bacterial infections, including contagious diseases that are life-threatening and broad-spectrum antibiotics have been widely used off-label for prophylactic treatment in food-producing animals (Carattoli, 2008).

Antimicrobial food producing animals has been shown to lead to the emergence of resistant bacteria throughout the food chain as the use of low doses of antibiotics by the modern food animal industry as growth-promoting substances in farm animals to promote animal growth and to prevent infections rather than cure infections is most likely responsible for drug-resistant bacteria emerging on farms (Meyer *et al.*, 2012).

The rapid emergence of extended-spectrum beta lactamases (ESBLs) in the food producing animals has been recorded and published worldwide (Horton *et al.*, 2011). It is important to its impact on the treatment and therapeutic strategy of serious infections (Hiroi *et al.*, 2011) as the occurrence of ESBLs in food producing animals has important implications for public health since these resistant determinants that are clinically relevant can be transmitted from animals to humans through either acquisition via the food chain (Smet *et al.*, 2008) or direct transmission to farm workers (Moodley and Guardabassi, 2009).

*Corresponding author:
Itamanvivien@yahoo.com.; **Itaman, V.O[1]**

Materials and Methods

Sample collection

A total of 45 faecal samples of pigs were collected from Oke-aro piggery farm at Oke-aro, Ogun state and University of Lagos piggery farm, Akoka, Lagos state in April, 2015. Samples were collected from pigs of different ages, sexes and breed. Animals under the study were reared under different housing systems and belonged to organized farms. The samples were collected with the aid of sterile swab sticks, placed in transport media (peptone broth) and conveyed to the laboratory for processing at the University of Lagos Microbiology Laboratory, Akoka, Lagos state. All the culture media were prepared according to the manufacturer's instructions. They were autoclaved at 121°C for 15minutes and glass wares were sterilized in dry air oven at 160°C for 1 hour.

Microbiological analysis

All samples collected were cultured primarily on MacConkey agar (LabM, UK) plates and also, on Salmonella-shigella agar (LabM, UK) and incubated at 37°C for 18-24hours. After incubation, the plates were examined for the production of pink colonies which shows the presence of lactose fermenters while the non-lactose fermenters appeared as translucent colourless colony with and without black centers. The lactose fermenters were repeatedly sub-cultured onto nutrient agar to obtain pure cultures. The pure isolates were stored in cryovials with nutrient agar in the refrigerator at 4°C. Biochemical tests according to CLSI (2010) such as motility-indole-urease (Rapid lab, UK) test, triple sugar iron (Rapid lab, UK) agar test and kligler iron agar (Rapid lab, UK) test were carried out for phenotypic identification (Cowan, 1993).

Antimicrobial Susceptibility Testing

Antibiotics susceptibility testing of the isolates was determined by the Kirby-Bauer disc diffusion method and interpreted according to the Clinical and Laboratory Standard Institute guidelines (CLSI, 2010). The antibiotic discs used were produced by Abtek Biological limited. The bacterial isolates were streaked on nutrient agar (Rapid Lab, UK) and incubated at 37^{0}C for 18-24 h. The discrete colonies obtained were inoculated into peptone broth and standardized to 0.5 McFarland. A sterile swab stick was dipped into the broth containing organisms and inoculated on Muller-Hinton agar (Oxoid, England) plates by rotating in order to ensure uniform distribution. The antibiotic sensitivity multi-discs were placed on the inoculated media with the aid of sterile forceps. The plates were incubated for 18 – 24 hours, after which the zones of inhibition were measured and recorded (Cheesbrough, 2006).

Double Disc Synergy Test (DDST)

Extended spectrum beta lactamase production detection in suspected isolates was determined by the DDST method which was performed by placing a combination disc of Amoxicillin/Clavulanic acid (20/10 µg) on the center of a Mueller-Hinton agar plate previously inoculated with the test organism. Cefotaxime (30 µg) and Ceftazidime were placed 25mm apart from the central disc, and the plates incubated at 37°C for 18-24 hrs. Enhancement of the inhibition zone towards the Amoxicillin/Clavulanate disc, indicating synergy between clavulanic acid and any one of the test antibiotics was regarded as positive for ESBL production (Spanu *et al.*, 2006).

RESULTS

Table 1.1: Frequency of isolation

Organisms	Number of isolates	Occurrence (%)
Citrobacter freundii	7	26.9
Salmonella spp.	6	23.1
Shigella spp.	1	3.9
Enterobacter aerogenes	2	7.7
Klebsiella pneumonia	5	19.2
Escherichia coli	5	19.2
Total	26	100

Table 1.2: The profile of five (5) *Klebsiella pneumonia*, one (1) *Shigella* spp. and six (6) *Salmonella* spp. isolates and the percentage (%) resistance to eight (8) antibiotics

Antibiotic disc Potency (µg)	No. and percentage (%) (*Klebsiella pneumonia)*		No. and percentage (%) *(Shigella* spp.*)*		No. and percentage (%) *(Salmonella* spp.*)*	
	S	R	S	R	S	R
Ceftazidime (30)	3(60)	2(40)	1(100)	0(0)	3(50)	3(50)
Nitrofuration (30)	4(80)	1(20)	0(0)	0(0)	6(100)	0(0)
Cefuroxime (30)	3(60)	2(40)	1(100)	0(0)	3(50)	3(50)
Gentamicin (10)	3(60)	2(40)	1(100)	0(0)	6(100)	0(0)
Cefixime (5)	3(60)	2(40)	1(100)	0(0)	6(100)	0(0)
Ofloxacine (5)	3(60)	2(40)	0(0)	0(0)	6(100)	0(0)
Augmentin (30)	3(60)	2(40)	1(100)	0(0)	6(100)	0(0)
Ciprofloxacin (5)	3(60)	2(40)	1(100)	0(0)	3(50)	3(50)

Table 1.3: The profile of (2) *Enterobacter aerogenes*, five (5) *E.coli* and seven (7) *Citrobacter freundii* and the percentage (%) resistance to eight (8) antibiotics

Antibiotic disc Potency (µg)	No. and percentage (%) *(Enterobacter aerogenes)*		No. and percentage (%) *(E.coli)*		No. and percentage (%) *(Citrobacter freundii)*	
	S	R	S	R	S	R
Ceftazidime (30)	0(0)	2(100)	3(60)	2(40)	5(71.4)	2(28.6)
Nitrofuration (30)	1(50)	1(50)	3(60)	2(40)	3(42.9)	3(42.9)
Cefuroxime (30)	0(0)	2(100)	2(40)	3(60)	2(28.6)	2(28.6)
Gentamicin (10)	2(100)	0(0)	3(60)	2(40)	3(42.9)	3(42.9)
Cefixime (5)	0(0)	2(100)	2(40)	3(60)	3(42.9)	3(42.9)
Ofloxacine (5)	2(100)	0(0)	3(60)	1(20)	2(28.6)	3(42.9)
Augmentin (30)	1(50)	1(50)	2(40)	3(60)	5(71.4)	1(14.3)
Ciprofloxacin (5)	1(50)	1(50)	4(80)	1(20)	5(71.4)	2(28.6)

The highest percentage occurrence of 26.9% was observed in the isolate *Citrobacter freundii*, and *Shigella* spp. had the lowest percentage occurrence of 3.9%. While *Salmonella* spp. had percentage occurrence of 23.1%, *Klebsiella pneumonia* and *E. coli* had same percentage occurrence of 19.2% and *Enterobacter aerogenes* had percentage occurrence of 7.7% (Table 1.1).

Shigella spp. was not resistant to any of the antibiotics, while *Klebsiella pneumonia* was resistant to all the antibiotics (Ceftazidime, Cefuroxime, Gentamicin, Cefixime, Ofloxacin, Augmentin and Ciprofloxacin) at a percentage of 40 except Nitrofuration at 20% (Table 1.2). *Salmonella* spp. was resistant to only Ceftazidime, Nitrofuration and Ciprofloxacin at a percentage of 50 each (As presented in table 1.2) and *Enterobacter aerogenes* to Ceftazidime, Cefuroxime, Cefixime at 100% and Nitrofuration, Augmentin and Ciprofloxacin at 50%. Also, *E. coli* and *Citrobacter freundii* were resistant to all the antibiotics at various percentages as shown in table 1.3 above.

Double disc synergy test (DDST) was used to assess ESBL production in twelve (12) isolates that showed multi drug resistance which are: *Citrobacter freundii*, *E. coli*, *Klebsiella pneumonia*, and *Enterobacter aerogenes* and none was found to be ESBL positive showing no synergy between Amoxicillin/Clavulanic acid and any of the test antibiotic used (Cefotaxime or Ceftazidime).

Discussion

In this study, the major finding is the presence of multi drug resistant *E. coli* and *Klebsiella pneumonia* to commonly used antibiotics such as augmentin, ceftazidime, cefixime, and cefu roxime. This observation reiterates the findings in other studies that have reported antibiotic resistance among bacteria (Ajayi *et al.*, 2011). The antibiotic susceptibility testing reveals common and disturbing patterns with high resistant rates for some of the isolates investigated as they were resistant to more than two of the tested antibiotics which defined them as multidrug resistant strain. The practice of the sales and uses of veterinary drugs such as augmentin without much control in piggery production in Nigeria could have resulted to a population of resistant bacteria in the animals (Aibinu, *et al.*, 2003). However, all the isolates showing multiple antibiotic resistances were screened for possible ESBL production using the double disc synergy test (DDST) and at the end, none was found to be ESBL positive. This is in line with the observation of Patterson that the in-vitro detection and identification of ESBLs is difficult and problematic (Patterson, 2003) and is affected by a lot of factors.

This could be due to the presence of high level of other antibiotics such as carbepenems and AmpC - β-lactams other than ESBLs which prevent recognition of ESBLs leading to false negative results (Rice, 2000). There are numerous reports such as those by Thompson in 2001, where *K. pneumonia* or *E. coli* isolates have been found to harbour plasmid mediated AmpC – type β- lactamases while some of these organisms harbour and co-express both AmpC – type β- lactamases and ESBLs (Thomson, 2001, Tzouvelekis, 1999). The presence of AmpC – β- lactamases resist inhibition by clavulanate and hence obscure or mask the synergistic effect of clavulanate and cephalosporins against ESBLs resulting in false negative test for the detection of ESBL. This has been shown in previous studies by Bush *et al.*,1993, where it was stated that the inhibitory effects of tazobactam against ESBL and AmpC beta-lactamase is greater than clavulanic acid by almost tenfold (Bush *et al.* 1993; Phillippon *et al.* 2002)..

It may also be one of the limitations of using ESBL confirmatory tests based on DDST in that they can yield false negative results with isolates that produce a high level of AmpC (Anderson, 2007). Recent studies by Odumosu *et al.* 2016, documented that their study was carried out using a modified method for DDST that included a fourth generation cephalosporin (cefepime) and tazobactam. The Cefepime is resistant to the production of AmpC beta lactamase that always masks the phenotypic detection of ESBL while the tazobactam acts as the beta-lactamase inhibitor (Tzelepi *et al.* 2000). Thomson

further buttresses this point by saying that tazobactam and sulbactam can serve as useful alternative agents for ESBL as they are less likely to induce the detection of high-level of AmpC beta-lactamases (Thomson, 2001). In addition, some antibiotics such as class A carbapenemases are not reliably detected by clavulanate – based methods but with more sensitive methods such as Modified Hodge Test (MHT) and Tris / EDTA – bases and boronic acid based tests (Anderson, 2007).

Other factors that may be responsible for the under-detection of ESBL production include; the presence of different antibiotic resistant mechanisms being harboured by these organisms against the antibiotics investigated (Collignon, 2012) or the inability to express the resistance gene and undetectable quantities of the gene being expressed (Essack, 2000).

Conclusion/ Recommendation

The results from this study demonstrated the high antimicrobial resistance amongst *E. coli* and *Klebsiella* spp. to the commonly prescribed antimicrobial drugs which substantiates the alarming occurrence and ongoing spread of various multi resistant *Enterobacteriaceae* strains in the human and animal population. To solve this problem, there is therefore, a need for continuous surveillance of antimicrobial resistance trends worldwide particularly among organisms resident in the gastrointestinal tract of farm-animals which are implicated in infectious diseases in human.

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