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DETECTION OF EXTENDED SPECTRUM OF β-LACTAMASE IN FEACAL *ESCHERICHIA COLI* FROM HUMAN, CATTLE AND CHICKEN IN ONDO STATE, NIGERIA

[1]ONIFADE, A. K, [1]INEGBU, U. O and [2]*FADIPE D. O.

Department of Microbiology, Federal University of Technology, P.M.B. 704, Akure, Ondo State, Nigeria. E-mail: profonifade@yahoo.com, Tel: +2348100090030
[2]Health Centre, Adekunle Ajasin University, Akungba-Akoko, Ondo State, Nigeria.
Corresponding Author: dipedare@gmail.com, Tel: +2348072905144

ABSTRACT

The presence of Extended Spectrum β-lactamase (ESBL) was assayed by the double disk synergy test using Amoxicillin-clavulanic acid, Cefoxitin, Ceftriaxone and Ceftazidime. Of the four antibiotics used against the 60 β-lactamase-producing *E. coli* isolates, 57 (95%) isolates showed the highest susceptibility/synergy to Cefoxitin, followed by Ceftriaxone 56 (93.3%), and Amoxicillin-clavulanic acid 49 (81.7%), while all the isolates were resistant to Ceftazidime (less 18 mm). Plasmid analysis carried out on all the ESBL -producing isolates showed that forty-five isolates were plasmid positive, while fifteen (15) isolates were chromosomal borne. Some of the isolates carried multiple plasmids, while some carried single plasmid. Twelve of the isolates each harboured a set of three plasmids. Four isolates harboured two plasmids each, while twenty-six isolates harboured single plasmids. However, the use of antimicrobial agents regarded as critically or highly important for use in humans should be avoided or minimized in food animals, to preserve the efficiency of these antimicrobial agents for the treatment of infection in humans.

Keywords: β-Lactamases, Plasmids, *Escherichia coli*, faecal samples.

INTRODUCTION

Escherichia coli becomes resistance to antibiotics through acquisition of the enzyme, beta-lactamase. Beta-lactamase genes are located on the large plasmids that confer resistance to other classes of antimicrobial agents and are readily transmissible from strain to strain and between different species of enteric Gram-negative bacilli (Schneider and Garrett, 2009).

Moreover, there is a probability of the spread of resistant strains from animals to humans, and thus healthy individuals can become carrier hosts for multiple antibiotic-resistant bacteria,

because humans consume these animal products (Reinthaler *et al.,* 2003; Wright, 2010). Adequate knowledge of the molecular basis of antibiotic resistance among enteric bacteria is of great importance as it will enable researchers to provide basic information concerning various mechanisms of bacterial resistance so as to control its spread when there is an outbreak. Antibiotic-resistant bacteria, particularly *Escherichia coli,* appear to be biologically fit and are capable of causing serious, life-threatening infections that are difficult to manage because treatment options are limited. Although researchers have shown that the incidence of antibiotic resistance among *Escherichia coli* is still on the increase. Hence, it is necessary to ascertain the degree of resistance among the strains of *Escherichia coli* recovered from some faecal samples, which may be reservoirs of the bacterium. The present study was designed to: (i) ascertain the *Escherichia coli* isolates that are β-lactamase producers through double disc synergy test (DDST) using third generation Cephalosporins; and (ii) isolate the plasmids of those *E. coli* isolates that showed multiple resistance to antibiotics

MATERIALS AND METHODS

Collection of samples

A total of nine hundred and fifty faecal samples were collected for this study. Three hundred samples were collected from different species of chicken: broilers, black layers and brown layers at the Teaching and Research Farm of Federal University of Technology Akure (FUTA). Faecal samples were also collected from students that were newly admitted to FUTA, babies that had diarrhoea at the Ondo State Specialist Hospital in Akure, and then from healthy and sick cattle at the abattoirs in Akure. Faecal samples were collected directly from the cloaca of chicken using sterile swab sticks in the early morning hours (6-7am). Faecal samples from diarrheic babies and apparently healthy adult humans were collected individually using sterile specimen bottles. All the samples were packed in properly labelled iced container for analysis in the laboratory within 1h of collection.

Isolation and characterization of bacteria

The samples were inoculated onto freshly prepared Eosin Methylene Blue (EMB) agar plates and incubated at 35ºC for 24h. Presumptive identification of *Escherichia coli* was based on the appearance of characteristic green metallic sheen on the EMB plates. A 1ml aliquot of

each dilution was plated onto MacConkey agar and eosin methylene blue agar, respectively. All the isolates were sub-cultured on nutrient agar plates and incubated at 37^{o}C for 24h. All the strains were further confirmed using standard biochemical techniques as previously described by Olutiola *et al*. (2001).

Extraction of plasmids

The plasmid extraction was carried out according to Kraft *et al.* (1988). A 1.5ml aliquot of overnight culture was spinned for 1 minute in a micro-centrifuge to pellet cells. The supernatant was gently decanted, leaving 50-100µl together with cell pellet; it was then vortexed at high speed to resuspend cells completely. Thereafter, a 300µl of TENS (Tris EDTA sodium hydroxide and SDS) was added and mixed by inverting tubes 3-5 times until the mixture became sticky. Then, 150µl of 3.0 sodium acetate with pH of 5.2 was added and vortexed to mix completely. The mixture was later spinned for 5mins to pellet cell debris and chromosomal DNA. Thereafter, the supernatant was transferred into a fresh tube and was mixed well with 900µl of ice-cold absolute ethanol. It was then spinned for 10minutes to pellet plasmid DNA.

Agarose gel electrophoresis

Agarose powder (0.8g) was mixed with 100ml of 1 x TBE (1 x Tris and Borate EDTA) buffer and dissolved by boiling for 30secs-1min at room temperature using a magnetic stirrer. It was allowed to cool to about 60°C and 10µl of ethidium bromide was mixed by swirling gently. The mixture was poured into electrophoresis tank with the comb in place to obtain a gel thickness of about 4-5mm, while bubbles were avoided. After leaving for about 20 minutes to solidify, the comb was removed and placed in electrophoresis tank. Then 1X TBE was poured into the tank ensuring that the buffer covered the surface of the gel. A 15µl aliquot of the sample was later mixed with 2µl of the loading dye and samples were carefully loaded into the wells created by the combs. After connecting the electrodes to the power pack, the electrophoresis was run at 60-100V until the loading dye migrated about three-quarter of the gel. Thereafter, it was turned off, the electrodes were disconnected and the gel was observed on a UV-transilluminator.

Statistical Analysis of Data

Data obtained from the study were subjected to statistical analysis of variance (ANOVA), while treatment means were compared using Duncan's new multiple range test (DNMRT) at 5% level of significance with SPSS version 16.

RESULTS

In this study, isolates of *E. coli* were presumptively identified as extended-spectrum-β-lactamase producers on the basis of their enhanced zone of inhibition on double disk synergy test (DDST) plates. Sixty ESBL-producing isolates were subjected to plasmid profile which detected the β-lactamase genes located on the plasmids as revealed by bands resolved by gel electrophoresis, (Plates 1a and 1b). Of the 60 β-lactamase producing *E. coli* isolates, 55 (91.7%) were susceptible to Cefoxitin, 53 (83.3%) isolates were susceptible to Amoxicillin clavulanic-acid while 49(81.7%) isolates were susceptible to Ceftriaxone, (Table 1). The distribution of the mean inhibition (synergy) zone diameters, around Cephalosporin discs with Amoxicillin clavulanic-acid, ceftriaxone 30mg, Cefoxitin 30mg and amoxicillin clavulanic-acid 30 mg, all showed synergy towards one another.

When plasmid analysis was carried out on the sixty (60) ESBL-producing *E. coli* isolates that were resistant to antibiotics to detect if such resistance was due to plasmid or chromosomal borne, forty-five (45) of the isolates were plasmid positive, while fifteen (15) isolates were chromosomal borne. Some of the isolates carried multiple plasmids, while some carried single plasmid. Twelve of the isolates each harboured a set of three plasmids (Lanes 6, 7, 11, 13, 15, 16, 17, 18, 19, 20, 21, 22). Four isolates (Lanes 2, 4, 5, 9) harboured two plasmids each, while twenty six isolates (Lanes 10, 12, 14, 23, 24, 25, 26, 27, 29, 30, 31, 33, 34, 35, 36, 39, 40, 42, 44, 45, 46, 49, 50, 51, 53, 54, 58, 59, 60) harboured single plasmids. However, isolates (1, 3, 8, 28, 32, 37, 38, 41, 43, 47, 48, 52, 55, 56, and 57) were chromosomal borne (Table 2).

Sixteen out of twenty isolates from apparently healthy human (adults) harboured plasmids and all the isolates from diarrheic babies were plasmid positive. Eight out of ten isolates from healthy cattle were plasmid positive and three out of five isolates from sick cattle were plasmid positive. Two out of four isolates from black layers were plasmid positive; three out of four isolates from brown layers were plasmid positive and five out of nine isolates from broiler harboured plasmids.

DISCUSSION

The poor susceptibility pattern of *E. coli* is attributed to the fact that *Escherichia coli* is among the organism that have the capability to produce β lactams enzymes, which are referred to as extended-spectrum-β-lactamases (ESBLs). Production of β-lactamases is the major mechanism through which bacteria develop resistance to antibiotics. By definition, extended spectrum βeta-lactamases are enzymes elaborated by organisms which inactivate penicillins and cephalosporins through hydrolysis of β-lactam core ring in the antibiotics (Daniels *et al.*, 2009). Though the number of *Escherichia coli* recovered from the sources used in this study, were not much as to compare to the number of samples analyzed, 60 (85.7%) of *Escherichia coli* isolated harboured the enzyme β-lactamase. ESBLs are found in a variety of Enterobacteriaceae species, of which *Escherichia coli* is a major ESBL producer (Nathisiuwan *et al*., 2001). The prevalence of ESBLs among members of Enterobacteriaceae constitutes a serious threat to the current β-lactam therapy, which leads to a frequent treatment failures and the resultant escalation of treatment costs (World Health Organization, 2002, 2007).

Plasmid analysis as carried out on the sixty ESBL producing isolates, revealed that 45 (75%) isolates harboured plasmids with different molecular sizes, while 15 isolates were chromosomal borne. Some of the isolates carried multiple plasmids, while some carried single plasmid. Twenty ESBL producing isolates from apparently healthy human harboured plasmids, and six out of the twenty harboured triple plasmid. This is an indication that the individuals must have consumed significant amounts of antibiotics unintentionally by eating the meat of animals to which they were fed or self- medication thereby leading to the increase in numbers of antibiotic-resistant bacteria which, when they invade the host, cause illnesses that are difficult to treat with antibiotics (Carman *et al.*, 2004). The resistant bacteria in animals due to antibiotic exposure can be transmitted to humans via three pathways; through the consumption of meat, from close or direct contact with animals, or through the environment. (Schneider and Garrett, 2009). However, it should be noted that complete cooking of meat inactivates bacteria, whether or not they are antibiotic-resistant.

The high prevalence of ESBL producers obtained from antibiotic resistance isolates in this study is a major cause for concern as this could be attributed to high rate at which organisms carry plasmids. Exposure to antibiotics is the most important risk factor for the development of resistance to antibiotics and the presence of extended spectrum β-lactamase genes among commensal bacteria in the gut of cattle and chicken facilitates the transfer of self-transmissible genetic elements within the gut (Oppegaard, 2001). Study by Shiraki *et al.* (2004) also reported a high frequency of transfer of plasmids carrying extended-spectrum β-lactamase genes among non-pathogenic *Escherichia coli* isolated from the gut of cattle in Japan.

The present work has further confirmed that extended spectrum β-lactamase producing *E. coli* in human and livestock is an emerging threat in the immediate study environment. However, the molecular characterization such as plasmid profile (analysis) assay has assisted in understanding the genetic basis of antibiotic resistance among commensal *Escherichia coli* isolated from feaces of human, cattle and chicken.

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Table 1: Zones of inhibition exhibited by some antibiotics against Extended Spectrum β-lactamase (ESBL) producing *Escherichia coli* isolates.

ESBL Producing Isolates from:	Zones of inhibition (mm)		
	Cefoxitin	Amoxicillin-Clavulanic acid	Ceftriaxone
Black Layers	22.12±0.05	27.09±0.3	19.09±0.02
Brown Layers	25.07±0.06	26.13±0.5	20.10±0.15
Broilers	23.31±1.1	24.67±0.2	23.44±0.08
Diarrheic Babies	22.09 ± 0.8	19.43±0.8	23.50±0.22
Healthy Cattle	28.03 ±0.3	26.80±1.0	19.11±0.11
Healthy Human	26.33 ±0.7	24.19±0.6	21.78±0.55
Sick Cattle	22.28 ±1.2	27.92±0.9	23.41±0.12

Values are means ± standard error

Table 2: Molecular weights and number of plasmids present in *Escherichia coli* isolates

S/N	*E. coli* Isolates	Base pair (unit of molecular weight of plasmid)	No of plasmid copies present
	Healthy Black Layer		
1	Nil	Nil	Nil
2	4743.6 bp	Base pair	Double plasmids
3	Nil	Nil	Nil
4	4566.09 bp	Base pair	Double plasmids
	Healthy Brown Layer		
5	Nil	Nil	Nil
6	4523.41 bp	Base pair	Double plasmids
7	94693.87 bp	Base pair	Triple plasmids
8	94693.87 bp	Base pair	Triple plasmids
	Healthy Cattle		
9	4527.41 bp	Base pair	Double plasmids
10	24451.71 bp	Base pair	Single plasmid
	Healthy Human		
11	94693.87 bp	Base pair	Triple plasmids
12	24451.71 bp	Base pair	Single plasmid
13	94693.87 bp	Base pair	Triple plasmids
14	24451.71 bp	Base pair	Single plasmid
15	94693.87 bp	Base pair	Triple plasmids
16	94693.87 bp	Base pair	Triple plasmids
17	94693.87 bp	Base pair	Triple plasmids
18	94693.87 bp	Base pair	Triple plasmids
	Diarrheic Babies		
19	94693.87 bp	Base pair	Triple plasmids
20	94693.87 bp	Base pair	Triple plasmids
21	94693.87 bp	Base pair	Triple plasmids
22	94693.87 bp	Base pair	Triple plasmids

23	24451.71 bp	Base pair	Single plasmid
24	24451.71 bp	Base pair	Single plasmid
25	24451.71 bp	Base pair	Single plasmid
26	24451.71 bp	Base pair	Single plasmid
	Healthy Cattle		
27	24451.71 bp	Base pair	Single plasmid
28	Nil	Nil	Nil
29	24451.71 bp	Base pair	Single plasmid
30	24451.71 bp	Base pair	Single plasmid
31	24451.71 bp	Base pair	Single plasmid
32	Nil	Nil	Nil
33	24451.71 bp	Base pair	Single plasmid
34	24451.71 bp	Base pair	Single plasmid
	Healthy Human		
35	24451.71 bp	Base pair	Single plasmid
36	24451.71 bp	Base pair	Single plasmid
37	24451.71 bp	Base pair	Single plasmid
38	24451.71 bp	Base pair	Single plasmid
39	24451.71 bp	Base pair	Single plasmid
40	24451.71 bp	Base pair	Single plasmid
41	24451.71 bp	Base pair	Single plasmid
42	24451.71 bp	Base pair	Single plasmid
43	24451.71 bp	Base pair	Single plasmid
44	24451.71 bp	Base pair	Single plasmid
45	24451.71 bp	Base pair	Single plasmid
46	24451.71 bp	Base pair	Single plasmid
	Sick Cattle		
47	Nil	Nil	Nil
48	Nil	Nil	Nil
49	24451.71 bp	Base pair	Single plasmid
50	24451.71 bp	Base pair	Single plasmid
51	24451.71 bp	Base pair	Single plasmid
	Broilers		
52	Nil	Nil	Nil
53	24451.71 bp	Base pair	Single plasmid
54	24451.71 bp	Base pair	Single plasmid
55	Nil	Nil	Nil
56	Nil	Nil	Nil
57	Nil	Nil	Nil
58	24451.71 bp	Base pair	Single plasmid

59	24451.71 bp	Base pair	Single plasmid
60	24451.71 bp	Base pair	Single plasmid

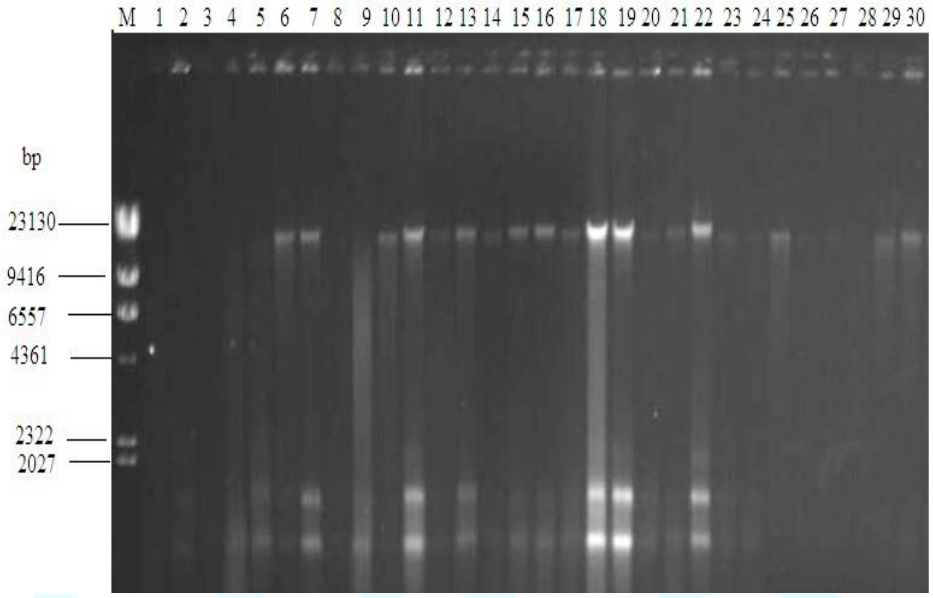

Plate 1a: Plasmid profile of ESBL producing *Escherichia coli* isolates in bands (Lanes 1-30).
M= Hind III DNA molecular weight marker
bp= Unit of molecular weight

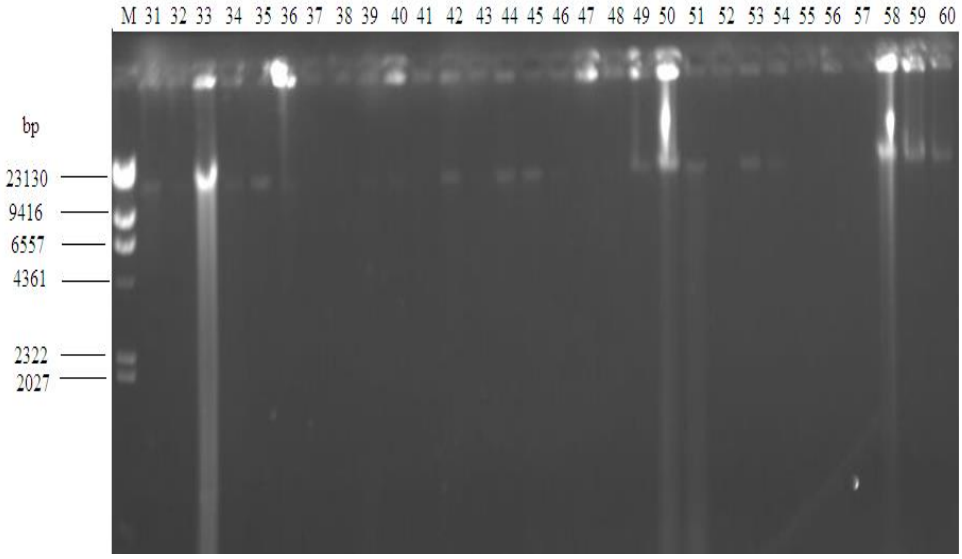

Plate 1b: Plasmid profile of ESBL producing *Escherichia coli*

isolates in bands (Lanes 31-60)

M= Hind III DNA molecular weight marker

bp= Unit of molecular weight